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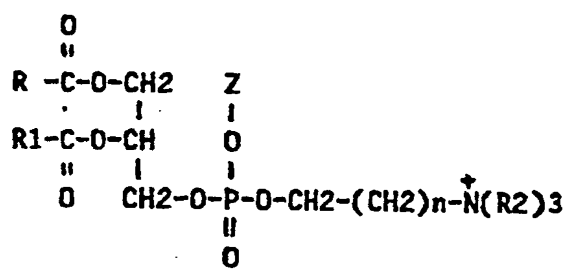
(54) Title: CATIONIC LIPID:DNA COMPLEXES FOR GENE TARGETING

(57) Abstract



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This invention herein describes pharmaceutical compositions and methods for targeted delivery of functional genes into cells and tissues in vivo. The invention discloses DNA:lipid complexes, methods of making such complexes and methods of using such complexes for facilitating the targeted delivery and entry of recombinant expression constructs into cells and tissues in vivo, and particularly delivery of such recombinant expression constructs to lung cells and tissues. The delivery vehicle for the targeting of recombinant construct encoding the gene of interest is composed of a mixture of a cationic lipid of formula (A): wherein Z is alkyl or alkoxyalkyl, n is an integer from 1 to 4 inclusive, R or R_1 are C_{11-29} straight chain aliphatic hydrocarbyl groups and R_2 is hydrogen or lower alkyl, together with neutral lipids (e.g. DOPE, cholesterol). The gene to be targeted is preferably CFTR gene for cystic fibrosis and the targeted tissue is lung.

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CATIONIC LIPID:DNA COMPLEXES FOR GENE TARGETING

BACKGROUND OF THE INVENTION

1. Field of the Invention

A perennial goal in the pharmacological arts has been the development of methods and compositions to facilitate the specific delivery of therapeutic and other agents to the appropriate cells and tissues that would benefit from such treatment, and the avoidance of the general physiological effects of the inappropriate delivery of such agents to other cells or tissues of the body. Recently, the advent of recombinant DNA technology and genetic engineering has provided the pharmacological arts with a wide new spectrum of agents that are functional genes carried in recombinant expression constructs capable of mediating expression of these genes in host cells. These developments have carried the promise of "molecular medicine," specifically gene therapy, whereby a defective gene could be replaced by an exogenous copy of its cognate, functional gene, thereby alleviating a variety of genetic diseases.

However, the greatest drawback to the achievement of effective gene therapy has been the limited ability in the art to specifically introduce recombinant expression constructs encoding functional genes into cells and tissues *in vivo*. While it has been recognized in the art as being desirable to increase the efficiency and specificity of administration of gene therapy agents to the cells of the relevant tissues, the goal of specific delivery has not been achieved in the prior art.

Liposomes have been used to attempt cell targeting. Rahman *et al.*, 1982, Life Sci. 31: 2061-71 found that liposomes which contained galactolipid as part of the lipid appeared to have a higher affinity for parenchymal cells than liposomes which lacked galactolipid. To date, however, efficient or specific delivery has not been predictably achieved using drug-encapsulated liposomes. There remains a need for the development of a cell- or tissue-targeting delivery system.

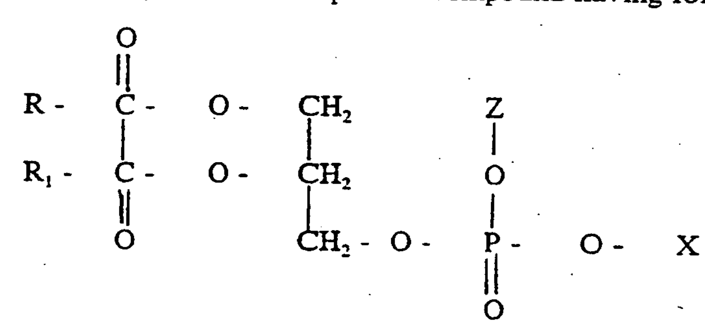
Thus there remains in the art a need for methods and reagents for achieving cell and tissue-specific targeting of gene therapy agents, particularly recombinant expression constructs encoding functional genes, *in vivo*.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to improved methods for targeted delivery of functional genes to cells and tissues *in vivo*. This delivery system achieves such specific delivery by the formation of DNA:lipid complexes between nucleic acid comprising a recombinant expression construct encoding a functional gene or fragment thereof complexed with a mixture of a cationic lipid and a neutral lipid. Methods of use are also provided. This invention has the specific advantage of targeted delivery of functional genes into cells *in vivo*, achieving effective intracellular delivery of constructs encoding functional genes more efficiently and with more specificity than conventional delivery systems.

In a first embodiment, the invention provides a pharmaceutical composition, comprising a formulation of a soluble complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid in a pharmaceutically acceptable carrier suitable for aerosol administration to an animal. In this embodiment of the invention, the recombinant expression construct comprises a nucleic acid encoding a transcription product, the nucleic acid being operatively linked to gene expression regulatory elements and whereby the nucleic acid is capable of transcription *in vivo*. In preferred embodiments, the recombinant expression construct encodes the human CFTR gene and is constructed to mediate efficient expression of the CFTR protein in lung epithelial cells. As used herein, the term "transcription product" is intended to encompass an RNA product resulting from transcription of a nucleic acid sequence, and explicitly includes RNA sequences that are not transcribed into protein (such as antisense RNAs or ribozymes), as well as RNAs that are subsequently translated into polypeptides or proteins.

In this first embodiment, the cationic lipid is a compound having formula I:



where Z is alkyl or alkylalkoxy, R and R_1 are independently straight-chain, aliphatic hydrocarbyl groups of from 11 to 29 carbon atoms, and X is a cationic moiety of formula

$$- CH_2 - (CH_2)_n - N^+(R_2)_3$$

where n is an integer from 1 to 4 inclusive and each R_2 is independently hydrogen or lower alkyl. In preferred embodiments, the cationic lipid is O-ethyl-dimyristoylphosphatidylcholine (EDMPC). In additional preferred embodiments, the neutral lipid is either cholesterol or dioleoylphosphatidylethanolamine (DOPE), and the EDMPC and cholesterol or DOPE are present in the complex at a ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct encoding human CFTR and a mixture of a neutral lipid and a cationic lipid comprises a molar ratio of DNA to lipid of 3:1 to 1:1 (μg DNA/ nmole lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the complex at a concentration of about 0.5-2.5mg/mL.

In a second embodiment, the invention provides a method for introducing a recombinant expression construct into a cell comprising lung tissue in an animal, the method comprising the step of administering the pharmaceutical composition of Claim 1 to the animal as an aerosol that is inhaled by the animal. In preferred embodiments, the recombinant expression construct encodes the human CFTR gene and is constructed to mediate efficient expression of the CFTR protein in lung epithelial cells. In preferred embodiments, the cationic lipid is O-ethyl-dimyristoylphosphatidylcholine (EDMPC). In additional preferred embodiments, the neutral lipid is either cholesterol or dioleoylphosphatidtlethanolamine (DOPE), and the EDMPC and cholesterol are present in the complex at a molar ratio of 1:1. Further preferred embodiments comprise a recombinant expression construct encoding human CFTR and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to lipid of 3:1 to 1:1 (μg DNA/ nmole lipid). Particularly preferred are embodiments where the DNA comprising the recombinant expression construct is present in the complex at a concentration of about 0.5-2.5mg/mL.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the

claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a histogram showing a comparison of luciferase activity in mouse lung tissue treated with luciferase-encoding plasmids complexed with EDMPC-Cholesterol, DDAB, water or encapsulated within an adenovirus vector delivery system.

Figure 2 is a graph of a comparison of chloride efflux in the presence and absence of stimuli in cells transfected with human CFTR-encoding plasmid vectors complexed with EDMPC:Cholesterol.

Figure 3 is a schematic representation of the plasmid pMB19.

Figure 4 is a graph of a comparison of chloride efflux in the presence and absence of stimuli in cells transfected with the human CFTR-encoding plasmid vectors pMB19 and pMB31 complexed with EDMPC:Cholesterol.

Figure 5 is a graph of chloride efflux in cells transfected with residual traces of human CFTR-encoding plasmid vectors pMB19 and pMB31 DNA/EDMPC:Cholesterol complexes as described in Example 4.

Figures 6 through 8 are photographs of agarose gel electrophoretic analysis of PCR products.

Figures 9 through 12 are photographs of *in situ* PCR results in primate lung.

Figures 13-22 are photographs of immunohistochemistry of primate lung sections showing human CFTR expression analysis results.

Figures 23 and 24 are graphs showing the extent of CAT activity, normalized to total protein, in heart, lung, spleen and pancreas of mice after intraperitoneal administration of p4119 DNA/EDMPC:cholesterol formulations of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions of matter and methods for facilitating the entry into cells of nucleic acids, particularly recombinant expression constructs encoding functional genes. For the purposes of this invention, the term

"recombinant expression construct" is intended to encompass a DNA construct comprising a nucleic acid encoding a functional gene or fragment thereof, operably linked to suitable control sequences capable of effecting the expression of the gene in a suitable host cell. Expressly intended to fall within the definition of a "gene" are embodiments comprising cDNA and genomic DNA sequences of functional genes, as well as chimeric hybrids thereof. Also intended to fall within the scope of the recombinant expression constructs of the invention are fragments or mutants of such genes which, when expressed, may inhibit or suppress the function of an endogenous gene in a cell, including, *inter alia*, *trans*-dominant mutants, antisense gene fragments and ribozymes.

In the recombinant expression constructs as provided by the present invention, the need for such control sequences will vary depending upon the host and cell types selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, optional or ancillary transcription control sequences, such as transcription factor binding domains, enhancer sequences, and other eukaryotic "operator" sequences to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. *See*, Sambrook *et al.*, 1990, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press: New York).

Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratible DNA fragments (i.e., fragments integratible into the host genome by homologous or non-homologous recombination). Also useful are vectors which replicate autonomously in host cells. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host cell.

The recombinant expression constructs of the present invention are useful in gene therapy, and specifically, for delivering exogenous, functional copies of a defective gene to a specific tissue target *in vivo*. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234.

The invention provides complexes of recombinant DNA constructs encoding

functional genes or fragments thereof and also comprising a mixture of a cationic lipid and a neutral lipid. For the purposes of this invention, the term "cationic lipid" is intended to encompass lipids which are positively charged at physiological pH, and more particularly, constitutively positively charged lipids comprising, for example, a quaternary ammonium salt moiety.

Specifically, the cationic lipids of formula I are O-esters of the acidic diacylphosphatidyl compounds and may be produced therefrom. In the cationic lipids of formula I, each of R and R_1 together with the carboxyl group to which they are attached, are straight-chain, aliphatic, hydrocarbyl acid moieties of from 12 to 30 carbon atoms inclusive, preferably from 15 to 25 carbon atoms inclusive. Such acid moieties are commonly referred to as fatty acid moieties, and may be saturated or ethylenically unsaturated and within the cations of formula I, R and R_1 are the same or are different. Illustrative moieties are lauroyl, myristoyl, palmitoyl, stearoyl, linoleoyl, tridecanoyl and oleoyl. In a modification where the cationic amphiphiles are prepared synthetically, it is advantageous for R and R_1 to be the same. Alternatively, when prepared from naturally occurring materials the R and R_1 moieties generally will be different.

Suitable Z groups are derived from alkanols or alkoxyalkanols which are straight-chain or branched. Illustrative Z groups include methyl, ethyl, propyl, isopropyl, n-butyl, sec-butyl, pentyl, hexyl, 2-methoxyethyl, 3-ethoxypropyl or 3-methoxypropyl. Preferred Z groups are straight-chain alkyl and more preferably the Z group is methyl or ethyl, especially ethyl.

Suitable X groups, illustrative by formula because of the complexity of the nomenclature include

$-CH_2-CH_2-N^+(CH_3)_3$,
$-CH_2-CH_2-N^+H_3$,
$-CH_2-CH_2-CH_2-N^+(CH_3)_2(C_2H_5)$,
$-CH_2-CH_2-N^+H_2CH_3$,
$-CH_2-CH_2-CH_2-N^+(CH_3)_3$ and
$-CH_2-CH_2-N^+H(CH_3)_2$

Preferred X groups are those wherein n is 1 and R_2 independently is hydrogen

or methyl. Cationic amphiphiles wherein at least one R_2 is other than methyl are novel.

Cationic lipids are particularly useful as carriers for anionic compounds, particularly polyanionic macromolecules such as nucleic acids. As cationic lipids are positively charged, a tight charge complex can be formed between a cationic lipid carrier and a polyanionic nucleic acid, resulting in a lipid carrier-nucleic acid complex which can be used directly for systemic delivery to a mammal or mammalian cell. Where delivery is *via* aerosolization, the charge complex will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:lipid carrier complex has been deposited in the lung following intranasal or intraoral delivery of the aerosolized complex.

Neutral lipids, in contrast to the cationic lipids of the invention, are characterized as being electrochemically neutral, although this definition does not preclude protonation of such lipids to produce a positively-charged salt under certain conditions. Expressly included within this definition are, *inter alia*, cholesterol and dioleylphosphatidylethanolamine (DOPE).

Complexes of DNA and mixtures of cationic and neutral lipids of the invention are characterized by a number of parameters intrinsic to the formation of such complexes. These include the identity of the cationic lipid and the neutral lipid; the ratio of cationic lipid to neutral lipid; concentration of DNA in the formulation; the ratio of DNA to lipid; DNA purity; cationic liposome size; the methods of preparing the DNA:lipid complexes; and other variables. Preferred combinations of cationic and neutral lipids include O-ethyl-dimyristoylphosphatidylcholine (EDMPC) and cholesterol and EDMPC and dioleylphosphatidylethanolamine (DOPE). Preferred molar ratios of these lipids is 1:1. DNA concentration in the complexes is from about 0.5mg/mL to about 5mg/mL, more preferably from about 0.5mg/mL to about 2mg/mL. DNA:lipid ratios are preferably from 1:1 to about 3:1 μg DNA/ nmole lipid, most preferably about 1:1 to about 2:1 μg DNA/ nmole lipid. DNA purity has a direct effect on liposome complex formation, but DNAs having a purity of about 15% to about 100% are appropriate for complex formation.

The various lipid carrier-nucleic acid complexes wherein the lipid carrier is a liposome are prepared using methods well known in the art. Mixing conditions can be optimized by visual examination of the resultant lipid-DNA mixture to establish that no precipitation occurs. To make the lipid-DNA complexes more visible, the complexes can be stained with a dye which does not itself cause aggregation, but which will stain either the DNA or the lipid. For example, Sudan black (which stains lipid) can be used as an aid to examine the lipid-DNA mixture to determine if aggregation has occurred. Particle size also can be studies with methods known in the art, including electronic microscopy, laser light scattering, Coulter™ counting/sizing, and the like. Standard-size beads can be included to calibrate instruments used for determining the size of any liposomes or complexes that form.

By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to the surface of a lipid carrier preparation, as discussed below. The lipid carrier preparation can also include other substances or cofactors. Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types. Generally, the nucleic acid material is added to a suspension of preformed liposomes which may be multi-lamellar vesicles (MLVs) or small unilamellar vesicles (SUVs), usually SUVs formed by sonication or by extrusion through appropriately-sized polycarbonate membranes. The liposomes themselves are prepared from a dried lipid film that is resuspended in an appropriate mixing solution such as sterile water or an isotonic buffer solution such as 10mM Tris/NaCl or 5% dextrose in sterile water and sonicated to form the liposomes. Then the preformed lipid carriers are mixed directly with the DNA. For delivery to the lung *via* aerosol or intratracheal delivery, the liposomes are preferentially about 100 μms in diameter.

Mixing and preparing of the lipid-DNA complex can be critically affected by the sequence in which the lipid and DNA are combined. Generally, it is preferable (to minimize aggregation) to add the lipid to the DNA at ratios of DNA:lipid from 6:1-5:1 through 1:1 inclusive (microgram DNA:nanomoles cationic lipid). Where the ratio of DNA:lipid is 1:4 or higher, better results are generally obtained by adding the DNA to the lipid. In either case, mixing should be rapidly achieved by shaking or vortexing

for small volumes and by use of rapid mixing systems for large volumes. The lipid carrier and DNA form a very stable complex due to binding of the negatively charged DNA to the cationic lipid carriers. The DNA:lipid complexes of the invention find use with small nucleic acid fragments as well as with large regions of DNA (≥30kb).

In preparing the lipid carrier-nucleic acid complex for nebulization, care should be taken to exclude any compounds from the mixing solution which promote the formation of aggregates of the lipid carrier-nucleic acid complexes. Large particles generally will not be aerosolized by the nebulizer, and even if aerosolized would be too large to penetrate beyond the large airways. Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/mL solution, and avoiding the use of chelating agents such as EDTA and/or significant amounts of salt, either of which tends to promote macro-aggregation. The preferred excipient is water, dextrose/water or another solution having low or zero ionic strength. Further, the volume should be adjusted to the minimum necessary for deposition in the lungs of the host mammal, while at the same time taking care not to make the solution too concentrated so that aggregates form. Increasing the volume of the solution is to be avoided if possible due to the need to increase the inhalation time for the host animal to accommodate the increased volume. In some cases, it may be preferable to lyophilize the lipid carrier-nucleic acid complexes for inhalation. Such materials are prepared as complexes as described above, except that a cryoprotectant such as mannitol or trehalose is included in the buffer solution which is used for preparation of the lipid carrier-DNA complexes. Any glucose generally included in such a buffer is preferably omitted. The lipid carrier is rapidly freeze-dried following mixing of the lipid and DNA. The mixture can be reconstituted with sterile water to yield a composition which is ready for administration to a host animal.

Complexes of the invention may be sized in accordance with conventional techniques, depending upon the desired size. In some instances, a larger complex injected into the bloodstream of an animal has higher affinity for lung cells as compared to liver cells. Therefore, the particular size range may be evaluated in

accordance with any intended target tissue and mode of administration by administering lipid-nucleic acid complexes of varying particle sizes to a host animal and determining the size of particle which provides the desired results.

The DNA:lipid complexes of the invention have utility in mediating the efficient delivery of the recombinant expression constructs of the invention, encoding functional genes or fragments thereof, into eukaryotic, preferably mammalian, most preferably human cells. DNA:lipid complexes of the invention are useful for achieving gene transfer *in vitro* using established techniques. More importantly, the DNA:lipid complexes provided by this invention, and the methods of administering the DNA:lipid complexes provided herein, are capable of specifically delivering recombinant expression constructs of the invention to particular tissues and cells comprising those tissues *in vivo*, thereby providing targeting of these genes to specific tissues. These properties of the pharmaceutical compositions and methods of the present invention provide for realization of practical gene therapy, whereby, *e.g.*, a particular deficient gene is restored by the introduction of a functional copy of the normal cognate gene into the cells of the affected tissue, without the inappropriate introduction of the construct into other cells and tissues of the body nonspecifically. In a particular embodiment, the present invention provides methods and pharmaceutical compositions for introducing a recombinant expression construct encoding the human cystic fibrosis transmembrane regulator (CFTR) gene into lung cells *in vivo*.

Thus, the invention provides methods and pharmaceutical compositions having a number of advantages over the prior art. The liposomes and lipid complexes of the invention have been extensively studied in humans, and are non-immunogenic, relatively non-toxic, and non-infectious. Recombinant expression constructs of any practicable size can be used, there being no limitation on large plasmid size due to the absence of packaging the DNA into the genome of a vector organisms like a retrovirus or an adenovirus. Gene transfer can be achieved in non-dividing cells, unlike prior art systems which relied on viral vectors whose life cycle required the infected cells to be dividing. In addition, the specific formulation of the DNA:lipid complexes of the invention can be altered to affect targeting and duration of the gene-expression

effect. The DNA:lipid complexes of the invention are also amenable to many delivery routes, and are less likely to encounter the types of safety issues related to viral-based delivery systems.

The DNA:lipid complexes of the invention have the advantage that the efficiencies of delivery are comparable with known prior art delivery systems. Figure 1 illustrates a comparison of the efficiency of delivery of a recombinant expression construct encoding firefly luciferase into mouse trachea using DNA:lipid complexes of the invention (EDMPC:chol) and an adenovirus-based vector system (Adeno-virus complex). The DNA:lipid complexes of the present invention show comparable expression to that seen with adenovirus, but without the inflammatory responses observed with adenoviral-based vectors.

The DNA:lipid complexes of the invention may be administered to an animal to effect delivery of functional genes into specific tissues by any appropriate therapeutic routine, including intravenous, intraperitoneal, subcutaneous, or intramuscular injection; direct injection into the target tissue(s); or, most preferably for the present invention, by aerosol delivery to the lung, using a nebulizer or other aerosol-producing device.

The methods and pharmaceutical compositions of the invention thus are particularly useful and appropriate for introducing functional human genes, particularly human CFTR, to lung tissue. These methods and pharmaceutical compositions thus have utility in the treatment of human diseases, including cystic fibrosis, asthma and chronic bronchitis.

The following Examples illustrate certain aspects of the above-described method and advantageous results. The following examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

Preparation of EDMPC:Cholesterol (1:1) Small Unilamellar Vesicles

To a 1L round bottom flask was added 500 μmoles cholesterol dissolved in an excess of chloroform and then 500 μmoles EDMPC were also dissolved in an excess of chloroform. The amount of EDMPC was determined by phosphorus assay (Bartlett,

1959, *J. Biol. Chem.* 234: 466; *Pharmacopoeial Forum* (1985) Nov.-Dec. 926; and (1986) Sept.-Oct. 1777) and not simply on the basis of the dry weight of the reagent.

After brief, gently mixing, the flask was attached to a rotary evaporating apparatus and chloroform withdrawn under slow speed and water vacuum conditions until almost all of the solvent was evaporated. Evaporation was completed at maximum rotation speed using a vacuum pump to completely dry the lipid mixture to a thin film on the wall of the round bottom flask.

As an intermediate step to the formation of the title composition, multilamellar vesicles (MLVs) were prepared from this film by the addition of 16mL endotoxin-free water to the flask, which was then warmed to 37°C in a water bath with gentle hand-swirling. The MLVs thus formed were removed from the flask using a 9" Pasteur pipette and transferred to a 20mm screw cap tube at room temperature. The flask was cleared of any remaining MLVs by washing with an additional 4mL endotoxin-free water, which was added to the 16mL previously transferred from the flask. These solutions were mixed, and aliquotted equally into 20 16mL screw cap tubes using a Pasteur pipette.

MLVs were converted into the SUVs of the title composition by sonication. Each of the 16mL screw cap tubes containing MLVs were placed individually into a sonicating water bath maintained at 36°C for 5 min, and the temperature of the bath checked between the introduction of each tube. Sonicated droplets within each tube were collected by brief vortex mixing, and the individual solutions of SUVs were then combined into a single 20mm screw cap tube using a 9" Pasteur pipette, and then filtered using a 0.2 micron disposable filter (Nalgene). Finally, an amount of an endotoxin-free solution of 25% dextrose in water, equal to one-quarter of the final volume of SUVs, was added to the tube of SUVs. This resulted in a suspension of SUVs comprising 20mM EDMPC and 20mM cholesterol (40mM total lipid) in a 5% dextrose solution, which was kept at 4°C until use.

EXAMPLE 2

Large Scale Plasmid DNA Preparation

Plasmid DNA was prepared in large-scale (*i.e.*, milligram) quantities using a

modification of the alkaline lysis procedure (Sambrook *et al.*, 1990, *ibid.*). Briefly, bacteria comprising a single colony were grown for 12-18 hours or overnight in 15mL TB broth (47g/L TB (Sigma Chemical Co., St. Louis, MO)/ 8% glycerol) supplemented with 100μg/mL carbenicillin at 37°C with shaking (250 rpm). 2-2.5mL of this culture was then added to 400mL TB (supplemented with 100μg/mL carbenicillin) in each of six 2L flasks (for a total of 2.4L culture) and grown at 37°C with shaking overnight (16-18h).

After overnight growth, bacteria were collected by centrifugation for 10 min. at 4°C in a Beckman J2-MI centrifuge equipped with a JA-10 rotor. The bacterial pellet in each centrifuge bottle was gently resuspended in 20mL of an ice-cold solution of 50mM dextrose in 25mM HCl buffer (pH8)/10mM EDTA. To the resuspended bacterial cell pellets were added 40mL of a freshly-made solution of 0.2N NaOH/1% sodium dodecyl sulfate at room temperature, resulting in cell lysis upon gentle agitation of this mixture on ice for about 5 min. After the added lysis solution has been thoroughly mixed into the bacterial suspension and the cells lysed, the mixture was allowed to stand at room temperature for 5 min. To this mixture of lysed bacteria was added 20mL of an ice-cold solution of 3M potassium acetate, which was mixed into the lysed bacterial solution gently by hand and then stored on ice for 10 min. A flocculate white precipitate formed, comprising bacterial chromosomal DNA, RNA and SDS/protein/membrane complexes, which were cleared from the solution by centrifugation at 8000rpm for 15 min at 4°C in the JA-10 rotor as above.

After centrifugation, the supernatant was transferred with filtering through Miracloth to 250mL centrifuge bottles, and 50mL isopropanol added at room temperature, mixed and incubated for 10 min. The plasmid DNA precipitate was recovered by centrifugation at 5000rpm for 10min at room temperature in a JA-14 rotor (Beckman). The alcohol-containing supernatant was decanted and residual supernatant removed by vacuum aspiration.

The plasmid DNA pellets were resuspended in 6mL of a solution of 6mM Tris-HCl (pH8) and transferred to 50mL centrifuge tubes upon dissolution. To each tube was added and equal volume of cold (-20°C) 5M LiCl, the solutions mixed by hand and then centrifuged at 8000rpm for 10min at room temperature in a JA-20 rotor

(Beckman). The supernatant solution from each tube was transferred to a fresh tube and the plasmid DNA then re-precipitated by the addition of an equal volume of isopropanol, mixed and collected by centrifugation at 5000rpm for 10min at room temperature in a JA-20 rotor. The alcohol-containing supernatant solution was then decanted, residual alcohol removed by aspiration, and the plasmid DNA pellets allowed to air dry for 5min.

Contaminating bacterial RNA was removed from the plasmid DNA by dissolving the pellets in 1mL 10mM Tris-HCl (pH8), adding about 0.5-0.75μg of pancreatic RNase per mL, followed by incubating the mixture at 37°C for 1h. Disappearance of RNA was determined by ethidium bromide-stained agarose gel analysis (*see* Sambrook *et al.*, *ibid.*). Plasmid DNA was purified by phenol-chloroform extraction. Briefly, to each aliquot of plasmid DNA solution was added an equal volume of Tris-saturated phenol:chloroform (1:1), the immiscible solutions mixed by vortexing, and centrifuged in a laboratory tabletop microfuge for 5min at room temperature. The aqueous (upper) layer was removed, transferred to a fresh microfuge tube, and extraction with phenol:chloroform repeated at least twice. These extractions were followed by two extractions of the aqueous layer with Tris-saturated chloroform. Plasmid DNA was concentrated by precipitation, with the addition of 5M sodium acetate to a final concentration of 0.3M and the addition of two volumes of cold (-20°C) absolute ethanol. DNA was allowed to precipitate in this solution at -20°C for 1h or overnight.

After precipitation, plasmid DNA was collected by centrifugation at about 6000rpm in a clinical microcentrifuge. The alcohol-containing supernatant was aspirated by vacuum, and the pellet washed twice with 70% ethanol/water (4°C). The washed pellets were air dried for at least 30min. Plasmid DNA pellets were dissolved in a total of 6mL of a solution of 10mM Tris-HCl (pH8), and concentration determined by spectrophotometric analysis of a 1-to-200 dilution of the recovered plasmid at A_{260}.

EXAMPLE 3

Preparation of DNA:Lipid Complexes

EDMPC:cholesterol:plasmid DNA liposomes were prepared as follows. An EDMPC:cholesterol mixture (1:1, 20μmoles/μL) was prepared as described in Example 1 above. Complexes with plasmid DNA were prepared in DNA:lipid ratios of 1:1 and 2:1 μgDNA/ nmole lipid; in preliminary experiments, unsuitable precipitates were observed to occur when DNA and lipid were mixed at ratios of 1:2 to 1:5. DNA and EDMPC:cholesterol were each first brought from storage conditions (-20°C for DNA, 4°C for liposomes) to room temperature before use over the course of about 1.5h. DNA concentration in the complex preparations were optimally 1000μg/mL complex solution (for ratios of 2:1 DNA:lipid) and 2000μg/mL complex solution (for ratios of 1:1 DNA:lipid). DNA concentrations were typically determined just prior to DNA:lipid complex formation, by ultraviolet spectrophotometry as described in Example 2. EDMPC:cholesterol mixtures were typically used at a concentration of 40μmole/mL total lipid, equivalent to 20μmoles/mL EDMPC and 20μmoles/mL cholesterol.

DNA:lipid complexes were prepared from these reagents as follows. Each component was prepared in individual microfuge tubes to a total volume per tube of 500μL. An appropriate amount of DNA (equivalent to a final DNA concentration of 1000μg DNA/mL complex) was added to one tube, and brought to volume with water or a solution of 5% dextrose in water. The appropriate amount of the EDMPC:Cholesterol mixture (500nmoles lipid/1000μg DNA at a 2:1 ratio; 2000nmoles lipid/2000μg DNA at a 1:1 ratio) was added to a second tube, and water or a solution of 5% dextrose in water was added to bring this solution to a total volume of 500μL. Each tube was mixed by vortexing for 15sec. The contents of the lipid mixture-containing tube were then added to the DNA-containing tube using a 1mL automatic pipettor. It was found that it was essential that this addition was performed slowly, in a constant stream, to the top of the DNA solution in tube A. As the lipid solution mixed with the DNA, formation of the DNA:lipid complex was detected by the solution becoming slightly cloudy and opalescent. It was also determined that, at this stage, the mixture could not be vigorously mixed (for example,

by vortexing) without seriously compromising the integrity and usefulness of the complexes so formed.

After the complexes were formed, the final concentration of DNA was determined by ultraviolet spectrophotometry as described above, and the size of the DNA:lipid complexes determined by light scattering measured at 400nm.

EXAMPLE 4

Detection of Functional CFTR Expression in Transfected Cells Using a Chloride Efflux Assay

A chloride ion efflux assay was used to detect functional expression of CFTR in transfected human 293 cells.

About 24h prior to introducing CFTR into 293 cells, cells were split into a 6-well tissue culture dish, each well receiving 1mL of 10mL of the cells on the dish and 3mL media. Cells were returned to the incubator and allowed to grow overnight at 37°C/5% CO_2, or until they were about 70-80% confluent. For assay, media were removed from the wells and each well was washed with 2mL serum-free media. 1mL of serum-free media was then added per well, and the cells incubated at 37°C for 1-2h. 200μl of a DNA-lipid complex comprising a recombinant expression construct encoding CFTR were then added to each well and incubated at 37°C for 6-8h. After this incubation, media were removed from each well, the wells were washed twice with 2mL serum-free media and incubated in 4mL serum-containing media at 37°C for 48h.

The chloride ion efflux assay was performed as follows. Media were aspirated from each of the wells containing cells treated with DNA-lipid complexes, and washed twice with efflux solution (135mM NaCl/2.4mM K_2HPO_4/0.6mM KH_2PO_4/1.2mM $CaCl_2$/1.2mM $MgCl_2$/10mM glucose/10mM HEPES (pH 7.4)). Cells were then incubated with 1mL efflux solution containing $Na^{36}Cl$ at a final concentration of 2.5μCi/mL $^{36}Cl^-$ for 2h at 37°C. After incubation, the $^{36}Cl^-$-containing efflux solution was aspirated from the cells and the cells then washed each of 4 times with 1mL efflux solution. The cells were then incubated with 1mL efflux solution for 3min at room temperature, and the efflux solution then removed from the cells and transferred into a scintillation vial containing 5mL scintillation cocktail. A fresh aliquot of efflux

solution was added to each well and incubated for an additional 3 min. After each incubation, efflux solution was transferred to a scintillation vial containing 5mL scintillation cocktail, and a fresh 1mL aliquot of efflux media was added to the cells and incubated for 3min. These steps of the assay were repeated ten times for a total of 30min. In certain of the wells, $^{36}Cl^-$ ion efflux was stimulated by incubating these cells in the presence of 40μM Forskolin (Sigma), 500μM cpt-cAMP (Sigma), and 100μM IBMX (Sigma) in efflux solution, efflux being stimulated at repetitions 3 through 7.

The extent of $^{36}Cl^-$ ion efflux over this period was determined by scintillation counting, and the basal rate of $^{36}Cl^-$ ion efflux compared with the rate of efflux in cells stimulated by Forskolin/cpt-cAMP/IBMX. Extent of efflux was normalized relative to the amount of $^{36}Cl^-$ ion remaining inside the cells after the 30min incubation. This quantity was determined by lysing the cells by incubating them with 1mL of scintillation fluid for 15min. The lysate from each well was then transferred into a scintillation vial, the well washed with 1mL of efflux solution which was added to the cell lysate, and the $^{36}Cl^-$ ion-associated radioactivity counted.

The results of one such assay are shown in Figure 2. Two plasmids encoding CFTR and differing in the details of the construct (*see* Table I) were tested with (closed circles and boxes) and without (open circles and boxes) stimulation. As is shown in the Figure, stimulation results in the rapid induction of chloride ion efflux over the basal rate of efflux, which efflux persists even after the stimulus is removed (time points 24-30). These results demonstrate the utility of this assay to detect functional expression of CFTR in heterologous cells, and thus forms an *in vitro* standard for determining the vigor of different recombinant expression constructs in expressing human CFTR.

EXAMPLE 5

<u>Functional Delivery of Human CFTR to Primate Lung Cells *In Vivo*</u>

Functional delivery of human CFTR into primate lung cells *in vivo* was

demonstrated using DNA:lipid complexes of CFTR-encoding plasmid DNA pMB19 (*see* Figure 3) complexed with EDMPC:cholesterol. Two rhesus monkeys were administered DNA/Lipid complexes by aerosol containing EDMPC lipid and hCFTR DNA complexes. The animals were euthanized 72 hours post-treatment and their respiratory airways evaluated for hCFTR expression. Rhesus monkeys were used for the simplicity of aerosol administration as well as their similarity to human lung physiology.

Two male rhesus monkeys (*Macaca mulatta*) were used. These primates were colony born at University of California, Davis Primate Research Center and examined prior to study start by a primate center veterinarian and were found to be in good health by clinical examination and laboratory studies. The animals weighed between 5.7 and 6.7 kg and each were administered Ivermectin (a parasiticide) at study start. Animals were individually housed during the study and lightly anesthetized before administration and necropsy. Animals were handled according to the NIH guide for the care and use of research animals.

Dosing of the animals was performed and necropsies done 72 hours post-administration. A MiniHEART nebulizer was used for the aerosol generation which was delivered via a tight-fitting face mask. Ten to 20 mL of DNA/lipid complexes were transferred to the nebulizer for each animal. Details of the experimental protocol are summarized in Table II below. Blood samples for clinical pathology and blood gas determinations were taken following anesthesia just prior to euthanasia. Animals were necropsied immediately following euthanasia. Harvested tissues and observations were confined to the respiratory tract.

TABLE I

Vectors with the CFTR cDNA

	enhancer	promoter	intron		polyA	antibiotic
MB19 :	HCMV	HCMV	ppi		ppi	amp
MB31:	HCMV	HCMV	ppi		SV40	amp
MB65:	HCMV	HCMV	ppi	Nmyc	ppi	amp
MB66:	HCMV	HCMV	ppi	Cmyc	SV40	amp
MB76:	HCMV	HCMV	ppi		3xSV40	amp
MB77:	---	CC10	ppi		3xSV40	amp
MB78:	HCMV	CC10	ppi		3xSV40	amp
MB81:	----	CFTR	ppi		3xSV40	amp
MB87:	HCMV	CFTR	ppi		3xSV40	amp
MB90:	HCMV	HCMV	---		3XSV40	amp
MB93:	HCMV	HCMV	pgl3		SV40	amp
MB97:	HCMV	HCMV	pgl3		SV40	amp/tet
MB113:	HCMV	HCMV	pgl3		SV40	tet

TABLE II

	Animal MMU25464	Animal MMU25744
Weight at Dosing (kg)	6.76	6.26
Time Period of Dosing (minutes)	150	84
Ventilation Fraction	39%	37%
Inhaled volume (L)	92.1	45.8
Aerosolized Liquid Total (mL)	15.3	10.1
Lung Deposition (65% of total liquid)* (mL)	3.1	1.8
Dosage (mL/kg)	0.46	0.29

*calculated volume

The cationic lipid, 1,2-dimyristoyl-*sn*-glycero-3-ethylphosphocholine, chloride salt (EDMPC, obtained from Avanti Polar Lipids, Inc., Alabama) was used to produce the DNA:lipid complexes used for aerosol administration. Liposomes were prepared by sonication of EDMPC and cholesterol (Sigma, Missouri) at 1:1 ratio as described above in Example 1. Preliminary testing of liposomes was done using a beta-galactosidase-encoding recombinant expression construct (pMB10; *see* Table I) for evaluation of the competency of these liposomes for *in vitro* transfection assay before making the dosing solution. These results showed that these EDMPC liposomes resulted in efficient gene delivery when complexed with DNA (data not shown).

CFTR-encoding plasmid DNA (pMB19; Table I) was used for complexing with these liposomes. The expression vector pMB19 contains the human cytomegalovirus (HCMV) promoter, the 5′ prepro-insulin intron, hCFTR cDNA, and the prepro insulin polyadenylation signal (see Figure 3). The vector also contains an ampicillin resistance gene for propagation in bacteria. Plasmid DNA was purified by alkaline lysis and phenol/chloroform extraction as described above in Example 2. HPLC analysis of plasmid DNA purity showed that these plasmid DNA preparations were between 13 and 27% pure plasmid DNA.

The dosing solution was comprised of DNA/lipid complexes containing

EDMPC:cholesterol liposomes and the hCFTR expression vector pMB19 at a 2:1 DNA:lipid ratio with a DNA concentration of 1mg/mL. Complexes were prepared as described in Example 3 above. A sample of each DNA/lipid complex was retained for evaluation in the chloride efflux assay to ensure potency, as described in Example 4 above. The results of this assay showed that the complexes were competent to produce functional hCFTR expression (see Figure 4). This assay was also used to evaluated the residual material remaining in the nebulizer after administration to the animals. This material was found to produce little if any efflux response (see Figure 5).

Animals were necropsied immediately following euthanasia 72 hours post-administration. Gross observations showed no significant lesions in any of the organ systems examined for animal MMU25744. Histological examination of animal MMU25744 showed a very mild pulmonary hemorrhage which appeared to be of the type associated with pulmonary hypertension. A lung mite infection appears to have caused peribronchiolitis. Gastrointestinal lesions seen were similar to those commonly seen in the UC Davis colony of animals. A very mild, multifocal acute hemorrhage in brainstem/cervical spinal cord appears to be associated with euthanasia. No other clinically significant effects were seen.

Animal MMU25464 (the higher does animal) had extensive hemorrhage involving the dorsal one-third of the right and left lungs. This hemorrhage extended into the parenchyma and was moderately more pronounced in the caudal lung lobes. Clotted blood partially filled the trachea and primary bronchi and the lungs failed to collapse. Total white cell count and blood gas determinations taken just prior to necropsy were normal, however, and the animal showed no signs of unusual clinical behavior. Similar to animal mMU25744, animal MMU25464, was observed to have granulomatous peribronchitis caused by lung mites, ischemic necrosis in the myocardium associated with euthanasia, and gastrointestinal lesions common to colony animals. Histological examination of regions demonstrated the accumulation of significant quantities of erythrocytes in airways and alveolar spaces. These were not accompanied by the presence of increased numbers of inflammatory cells nor any evidence of erythrophagocytosis. No other significant histological changes were recognized.

Samples for CFTR analysis were taken from the following tissues: pharynx, esophagus, mediastinal lymph node, trachea, right and left anterior lung lobe, right and left middle lung lobes, right and left caudal lung lobes, and right azygos lung lobe. The tissues were prepared as required for the assays designed to monitor delivery and expression of the pMB19 DNA described below. Tissues from two untreated primates were analyzed coincidentally with the transfected animals. These controls included a primate of the same species as well as from a different species (*Macaca fasicularis* for immunohistochemistry and *Macaca nemistrina* for *in situ* RT-PCR). Additional tissues were collected from treated animals for routine histological evaluation.

CFTR expression was assayed using a reverse transcriptase polymerase chain reaction assay (RT-PCR) on whole tissues. These assays were performed using vector specific primers and CFTR specific primers. The vector specific primers used were:

5′ AGA TCG CCT GGA GAC GCC AT 3′	forward primer (3651-3671bp in pMB19; SEQ ID No.: 1)

and

5′ GCT CCT AAT GCC AAA GGA AT 3′	reverse primer (1246-1266 bp in pMB19, upstream from hCFTR ATG site; SEQ ID No.: 2).

The CFTR specific primers were used:

5′ CCT GTC TCC TGG ACA GAA A 3′	forward primer (3337-3355bp in pMB19; SEQ ID No.: 3)

and

5′ GTC TTT CGG TGA ATG TTC TGA C 3′	reverse primer (3651-3671 bp in pMB19; SEQ ID No.: 4).

Tissues were frozen on dry ice for RT-PCR and stored at -70°C. Tissue samples were homogenized and used directly in this evaluation.

Briefly, RT-PCR was performed by preparing first-strand cDNA from cellular RNA isolated from frozen tissues using standard techniques (*see* Sambrook *et al.*, *ibid.*), including specifically the use of random hexamer for priming and MMLV-

derived reverse transcriptase. cDNA was used in PCR reactions performed as follows. The entire 25μL of the first-strand cDNA reaction was mixed with the components of the PCR reaction (under standard conditions; *see* Innis *et al.*, 1990, PCR Protocols: A Guide to Methods and Applications, Academic Press, New York), including 25μM apiece of each of the specific pairs of PCR primers. PCR reactions were overlayed with light mineral oil to prevent condensation and then subjected to the following PCR cycling protocol:

1 cycle	10min 94°C
30 cycles	1min 94°C
	2min 55°C
	3min 72°C
1 cycle	10min 72°C
	2min 27°C.

After completion of the reaction, the apparatus was programmed to take and hold the reaction mixtures at 4°C.

Results of these assays are shown in Figure 6. In these assays, the vector specific primers were expected to yield a band representative of plasmid DNA (485bp) and a hCFTR RNA-specific band (142bp). The results shown in Figure 6 revealed the expected plasmid DNA band but not the RNA band. Other bands are also present which appear to be due to non-specific priming.

The use of vector-specific primers was approximately 4-fold less sensitive in detection of hCFTR RNA than the CFTR specific primers. Therefore, RT-PCR was also performed on all tissues using the CFTR specific primers. In these experiments, a band of the expected size (334bp) was present in both the treated and untreated tissues (Figures 7 and 8). The intensity of this band was diminished when the untreated tissue samples were treated with DNase (data not shown). This decrease in the intensity of the band following DNase treatment indicated that the produced DNA fragment was, in part, due to PCR amplification of endogenous primate genomic DNA sequences, which produce a DNA fragment of the appropriate size even in the control (untreated) animals. The minor band which remains in the untreated tissues after

DNase treatment is removed by RNA treatment and reflects the very low level of endogenous CFTR transcription present in the control animals.

In situ RT-PCR was also performed using the CFTR specific primers as described above. Tissues were paraformaldehyde fixed, paraffin embedded and sectioned for *in situ* RT-PCR, using a slight modification of the procedure described by Nuovo (1992, PCR in situ Hybidization: Protocols and Applications, Raven Press, New York), hereby incorporated by reference herein.

In tissues from untreated animals analyzed by *in situ* RT-PCR, no signal was detected, indicating that within the limits of sensitivity of this assay, endogenous CFTR was not detectable in untreated animals (Figure 9). In contrast, a clear signal was observed in the cells lining the airway of the lungs in one of the treated animals (Figure 10). This signal was found to be dependent on the presence of RNA (it was destroyed by RNase treatment) and the addition of reverse transcriptase (no signal was obtained in the absence of first-strand cDNA synthesis). Controls using DNase with no reverse transcriptase (Figure 11) and DNase plus RNase treatment (Figure 12) consistently showed no staining.

The RT-PCR data suggest that the vector specific primers can detect CFTR DNA and CFTR specific primers can detect DNA and RNA in both treated and untreated tissues. The signal obtained using CFTR specific primers in untreated tissue indicates that endogenous CFTR was being detected. Differences in sensitivity of these primers preclude their use as a direct measurement of transfection in whole tissue RT-PCR. However, since no signal was seen in untreated tissues with CFTR specific primers for in situ RT-PCR, it appears that these primers are useful for *in situ* RT-PCR.

Immunohistochemistry was also performed for detection of the CFTR protein with commercially available antibody (Genzyme) and proprietary antibodies. Genzyme antibodies were reactive to the R domain of CFTR and the carboxyl terminus of CFTR. A proprietary antibody (MB1) was produced in rabbits using the last 13 amino acids of the C-terminus of CFTR, as described by Marino *et al.* (1991, "Localization of the Cystic Fibrosis Transmembrane Conductance Regulator in Pancreas," *J. Clin. Invest.* 88,: 712-716). Tissues were collected in OCT and frozen

in isopentane chilled in liquid nitrogen and stored at -70°C until sectioning for immunohistochemistry. Standard immunohistochemistry procedures were used with alkaline phosphatase conjugated to goat anti-rabbit as the secondary antibody. Levamisole was used to inhibit endogenous alkaline phosphatase.

In sections of the trachea, right middle lobe, right anterior lobe and right caudal lobe, staining was apparent in the cells lining the airways in the treated animals. For the purposes of illustration, selected sections from Animal MMU25744 are shown: trachea, right anterior lung lobe, right middle lung lobe and right caudal lung lobe (corresponding to Figures 13-16). Comparable results were obtained in animal MMU25464 as demonstrated in stained sections of trachea and right middle lung lobe (Figures 17-18). Untreated primates showed little to no staining in lung tissue (Figure 19). Other controls included: an irrelevant first antibody (anti-CAT) (Figure 20); incubation with second antibody without first antibody staining (Figure 21); and the addition of excess free peptide to absorb CFTR-specific antibody prior to staining (Figure 22). All controls were consistently negative.

Immunohistochemical analysis demonstrated transfection of the treated animals with staining apparent in the cells lining the airways. However, this assay is unable to assess the intracellular location of expressed CFTR protein. In samples from the treated animals, tracheal sections show 60-80% of the ciliated epithelial cells to be positive for CFTR protein with the apical regions of these cells staining most intensely. Immunostaining within the lung was confined principally to bronchi and bronchioles. Of these larger airways, 60-90% of the epithelial cells were positively stained. In some instances, staining within an individual airway was patchy with an abrupt transition between positively and negatively stained cells. Within the same section, a positively stained bronchiole and a closely adjacent negatively staining bronchiole could sometimes be seen. The high level of transfection appeared to diminish in the deeper regions of the lung, probably due to dosing limitations. At least 50% of the bronchi and bronchioles within a given section displayed significant staining. Alveolar macrophages in some sections also appeared to be staining; however, similar staining patterns can be seen in control sections. These results are substantially similar to the results obtained by *in situ* RT-PCR tissue section staining.

These results demonstrated functional delivery of a recombinant expression construct encoding human CFTR using the DNA:lipid complexes of the invention, and expression of human CFTR in primate lung tissues *in vivo*.

EXAMPLE 6

Functional Delivery of CAT-Encoding Plasmid DNA to Mouse Pancreas *In Vivo*

Functional delivery of the chloramphenicol acetyl transferase (CAT) expression vector p4119 (Figure 22) to pancreatic cells *in vivo* was demonstrated using DNA:lipid complexes of p4119 complexed with EDMPC:cholesterol. Complexes were prepared with EDMPC:cholesterol at molar ratios of 1:6, 1:1 and 2:1, and at a total DNA concentration of from 125-400μg/200μL formulation. 200μL of formulation were administered by intraperitoneal injection into each of 9 mice; four animals were injected with lipid complexes comprising 5% dextrose in distilled water rather than DNA.

Each animal was euthanized and heart, lung, spleen and pancreas tissued harvested. Total protein and CAT activity (determined as ^{14}C counts incorporated into acetylchloramphenicol as disclosed in Example 3 above) were determined, and the extent of CAT activity in each tissue calculated as cpm/mg protein. Results of these experiments are shown in Figures 23 and 24. These results demonstrate that formulations having a EDMPC:cholesterol molar ratios of 2: 1 to 1:1 were capable of specifically delivering CAT-encoding plasmid DNA to pancreatic tissue *in vivo*.

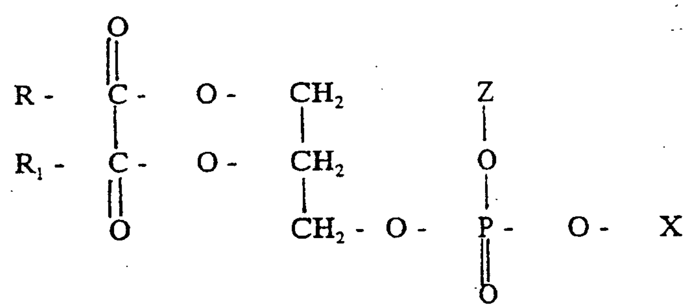
It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WE CLAIM:

1. A pharmaceutical composition comprising a formulation of a soluble complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid in a pharmaceutically acceptable carrier suitable for aerosol administration to an animal wherein

(a) the recombinant expression construct comprises a nucleic acid encoding a treanscription product and wherein said nucleic acid is operatively linked to gene expression regulatory elements whereby the nucleic acid is capable of transcription *in vivo* in lung epithelial cells; and

(b) the cationic lipid is a compound having formula I:



where Z is alkyl or alkylalkoxy, R and R_1 are independently straight-chain, aliphatic hydrocarbyl groups of from 11 to 29 carbon atoms and X is a cationic moiety of formula

$$- CH_2 - (CH_2)_n - N^+(R_2)_3$$

where n is an integer from 1 to 4 inclusive and each R_2 is independently hydrogen or lower alkyl.

2. The pharmaceutical composition of Claim 1 wherein the cationic lipid is O-ethyl-dimyristoylphosphatidylcholine.

3. The pharmaceutical composition of Claim 1 wherein the neutral lipid is cholesterol or dioleoylphosphatidylethanolamine.

4. The pharmaceutical composition of Claim 1 wherein the cationic lipid is O-ethyl-dimyristoylphosphatidylcholine and the neutral lipid is cholesterol or

dioleoylphosphatidylethanolamine.

5. The pharmaceutical composition of Claim 4 wherein the cationic lipid and the neutral lipid are present in a molar ratio of from about 3:1 to about 1:1.

6. The pharmaceutical composition of Claim 1 wherein the complex of a recombinant expression construct and a mixture of a neutral lipid and a cationic lipid comprises a ratio of DNA to lipid of 2:1 μg DNA/ nmole cationic lipid.

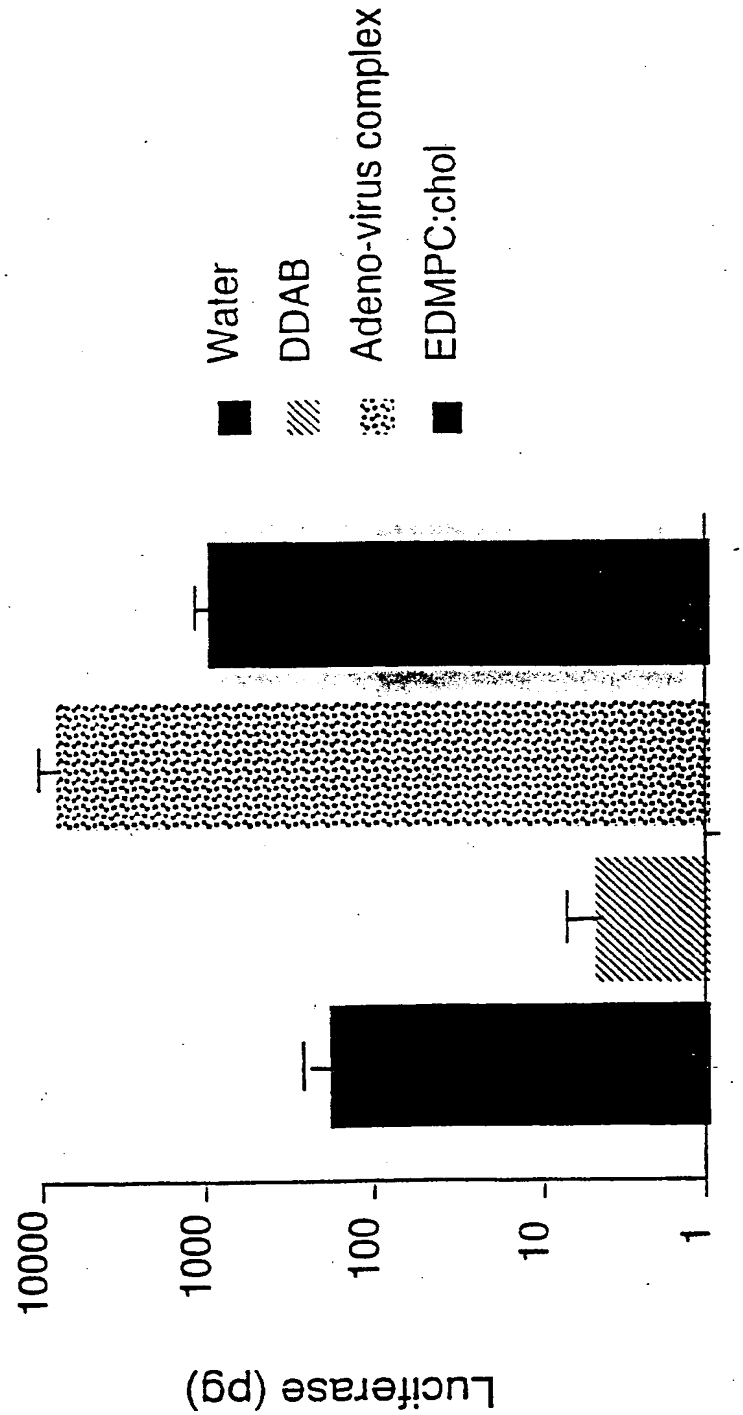
7. The pharmaceutical composition of Claim 1 wherein the nucleic acid comprising the recombinant expression construct is present in the complex at a concentration of about 0.5mg/mL to about 2mg/mL.

8. The pharmaceutical composition of Claim 1 that encodes human CFTR.

9. Use of the pharmaceutical composition of Claim 1 to prepare a medicament for introducing a recombinant expression construct into a cell comprising lung tissue in an animal wherein the pharmaceutical composition is administered as an aerosol that is inhaled by the animal.

10. A use according to Claim 9 wherein the recombinant expression construct encodes human CFTR.

Figure 1



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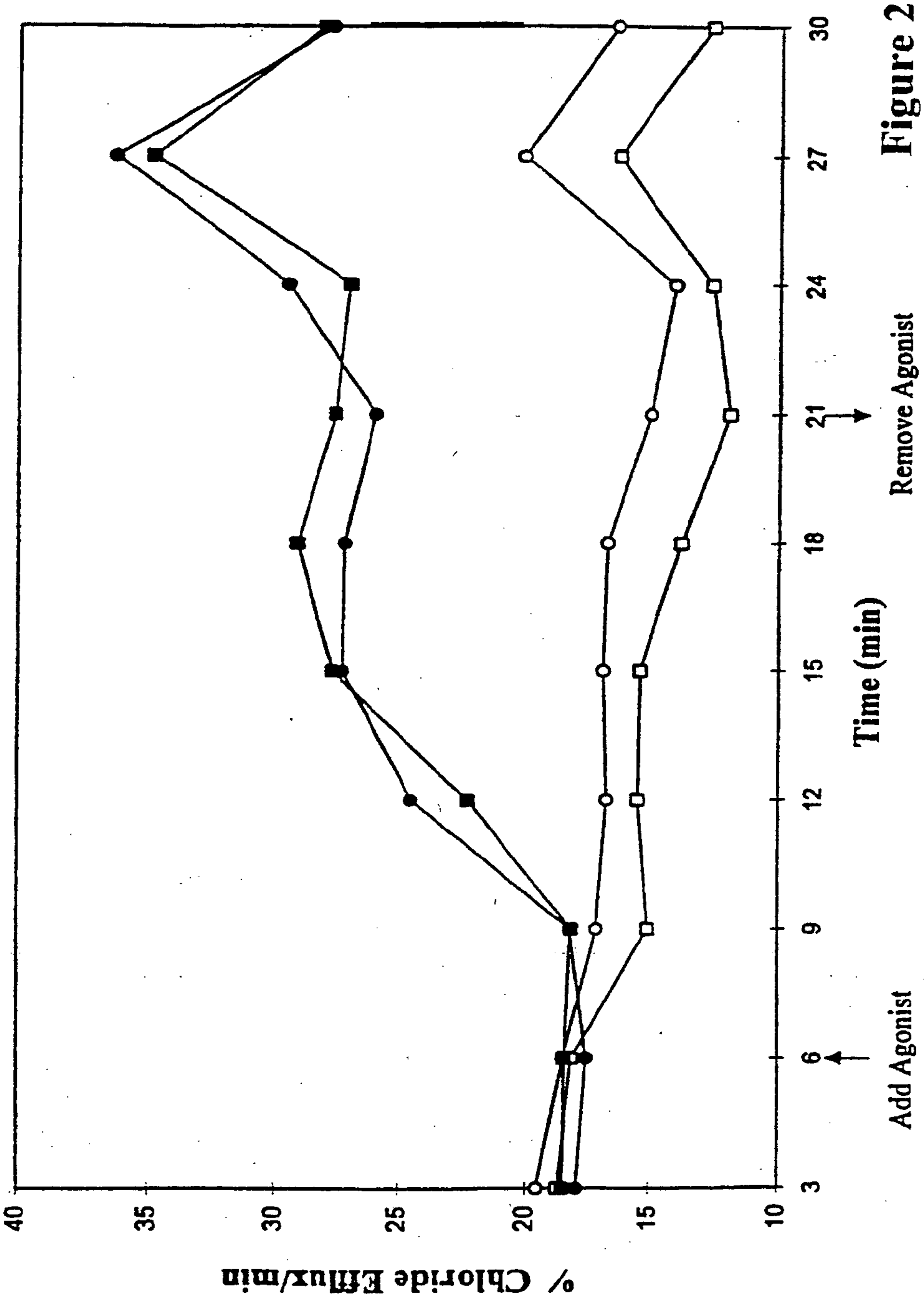

Figure 2

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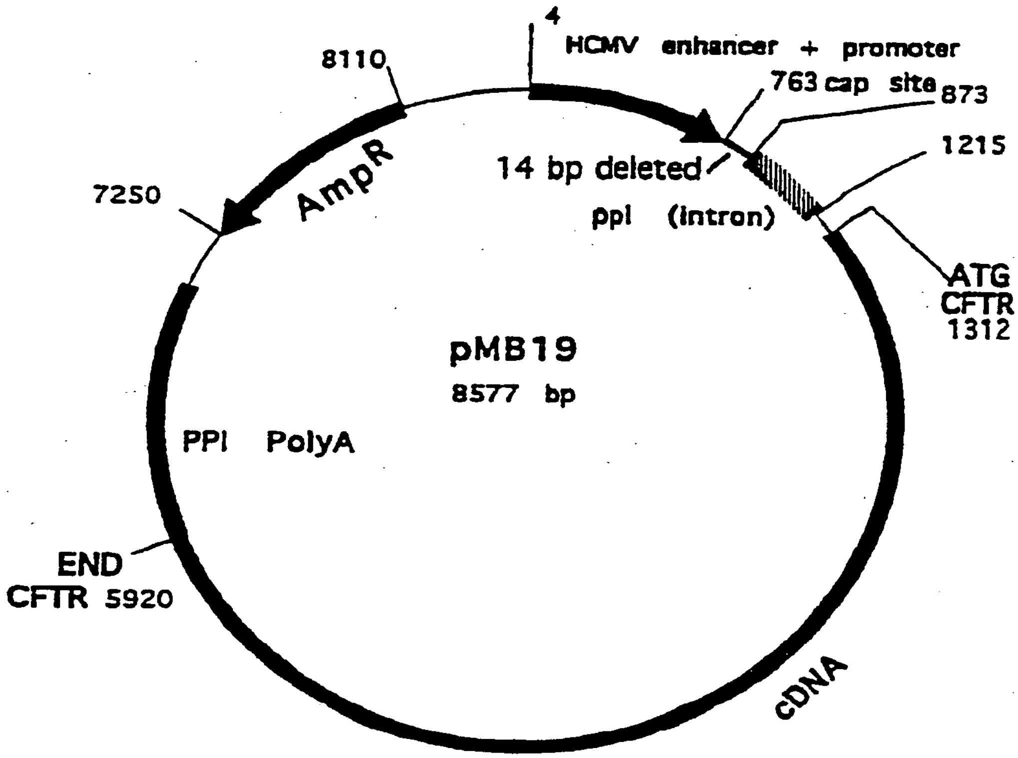
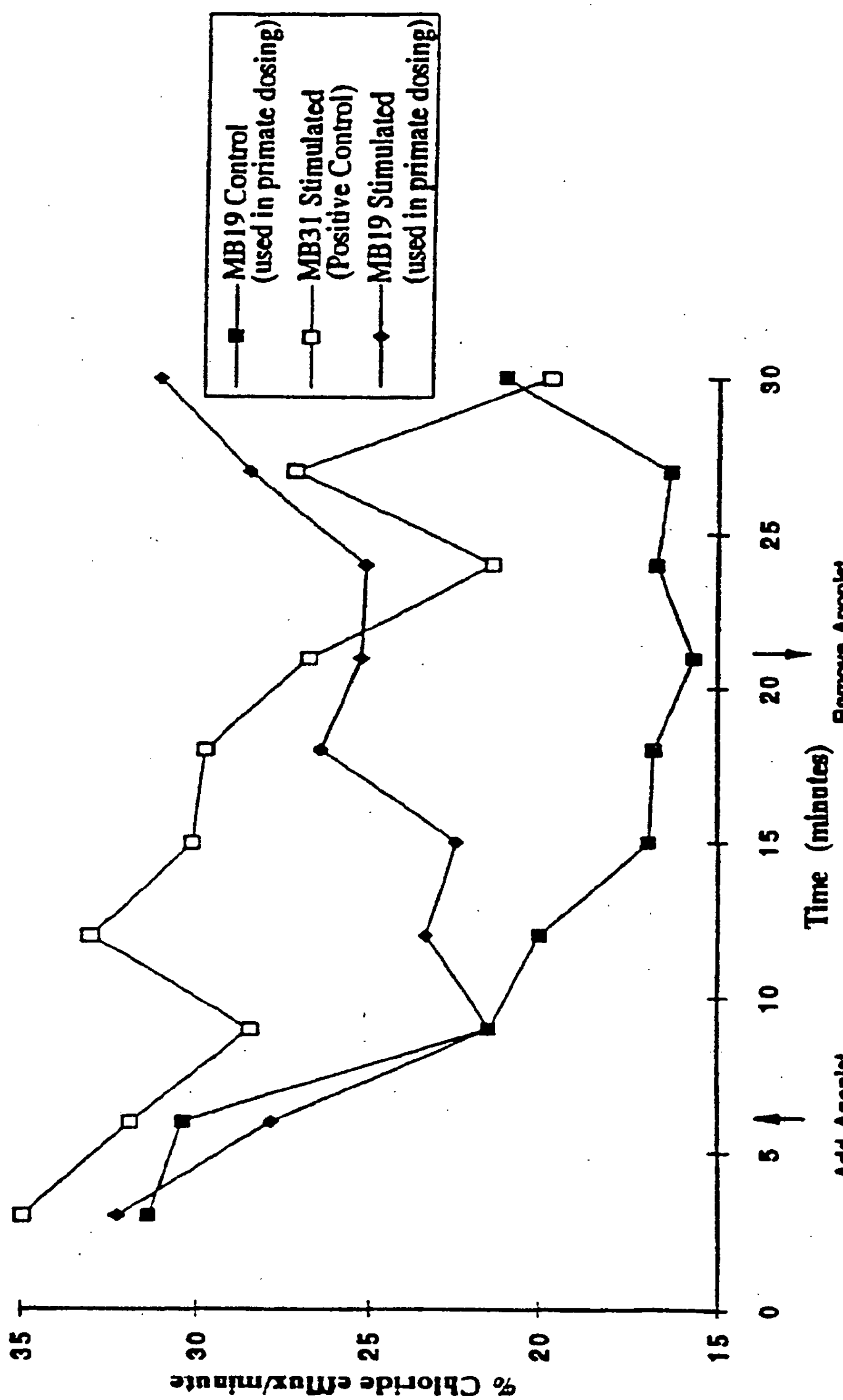

4
HCMV enhancer + promoter
763 cap site
873
1215
14 bp deleted
ppl (intron)
ATG
CFTR
1312
8110
AmpR
7250
pMB19
8577 bp
PPI PolyA
END
CFTR 5920
cDNA

Figure 3

Figure 4



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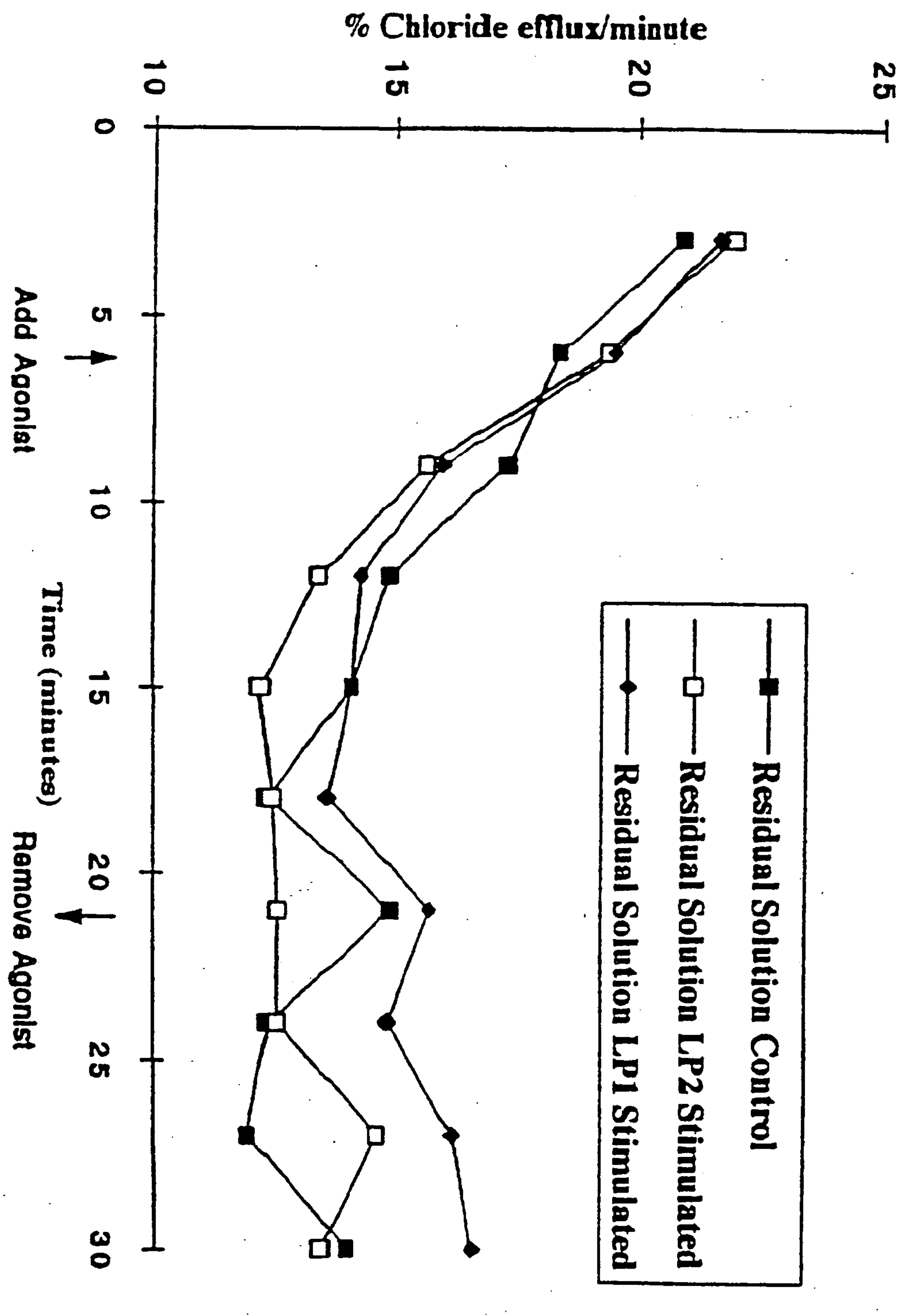

% Chloride efflux/minute
10
15
20
25
0
5
10
15
20
25
30
Time (minutes)
Add Agonist
Remove Agonist
Residual Solution Control
Residual Solution LP2 Stimulated
Residual Solution LP1 Stimulated

Figure 5

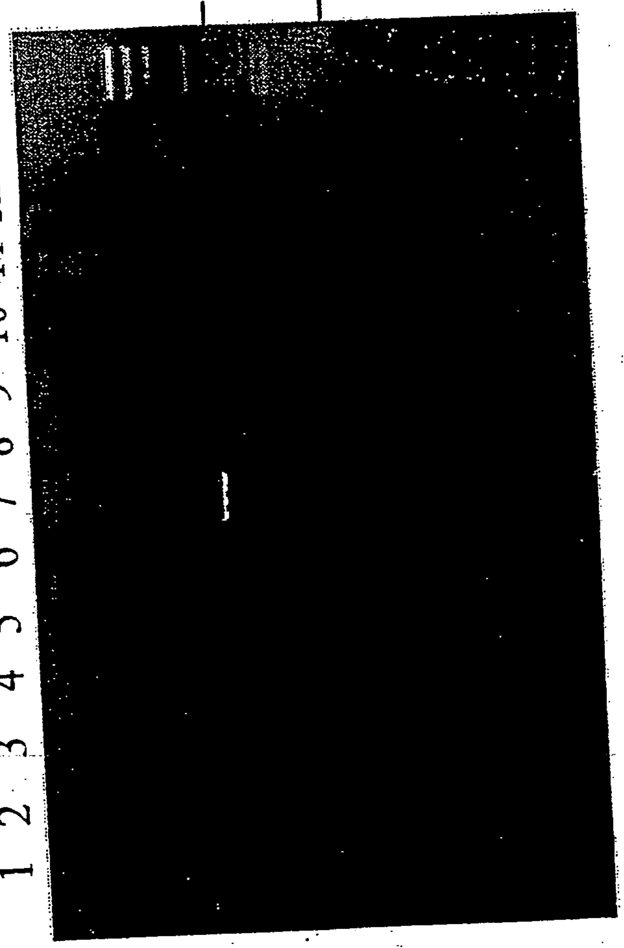


Figure 6

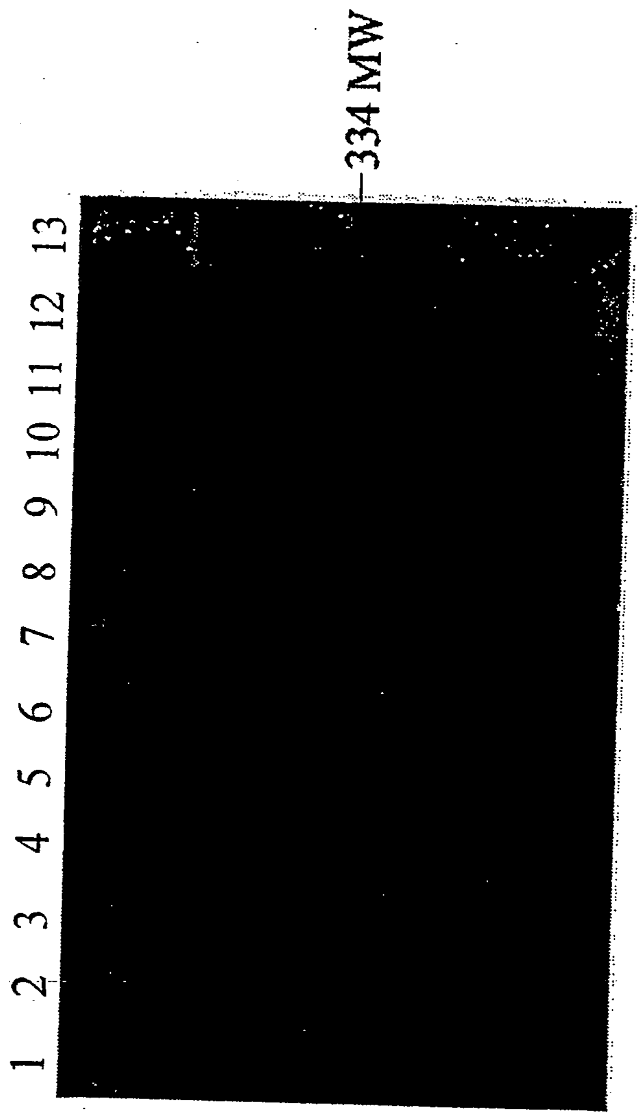


Figure 7

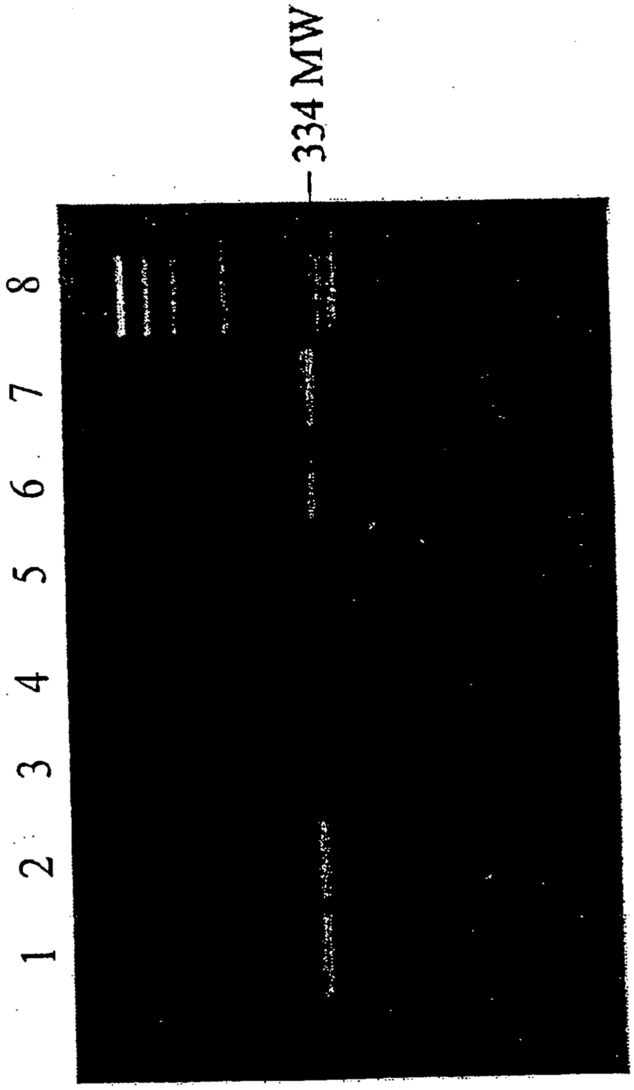


Figure 8

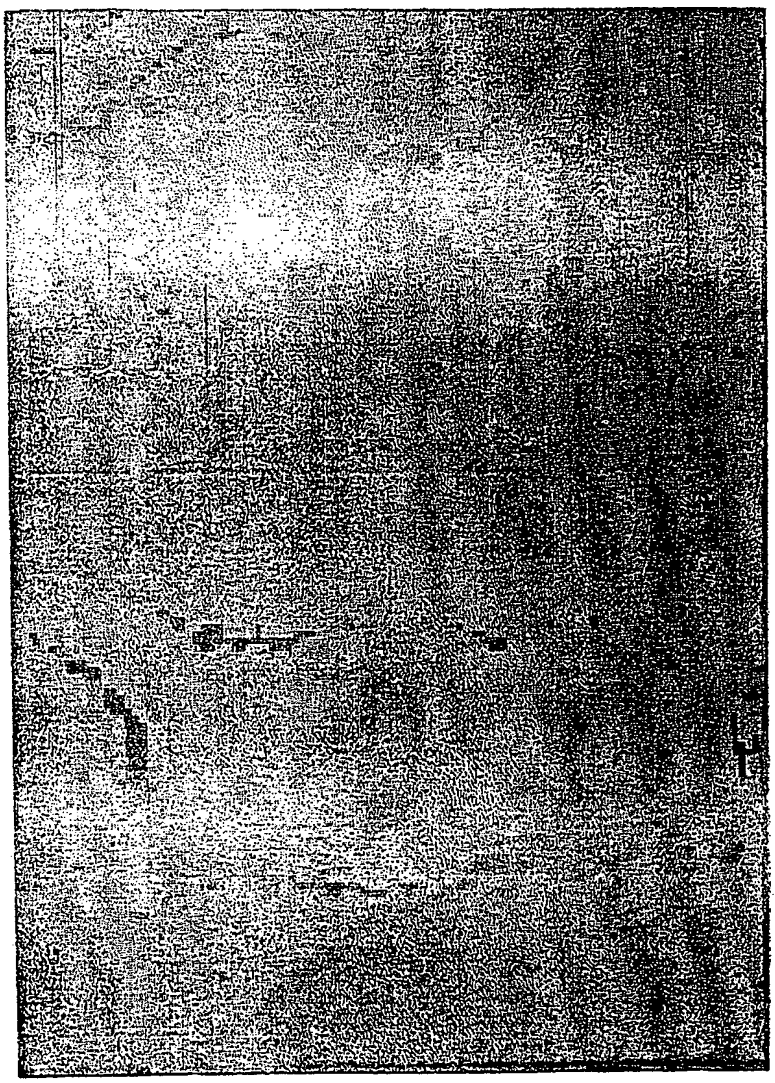


Figure 9

Figure 10

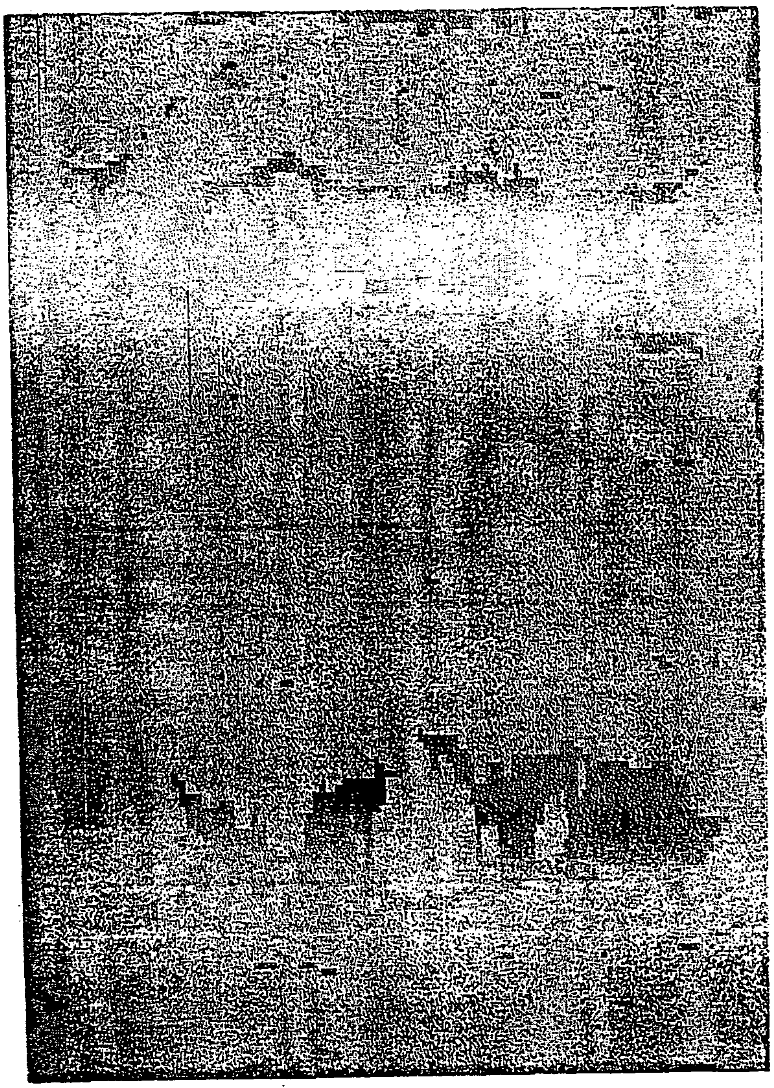


Figure 11

Figure 12

Figure 13

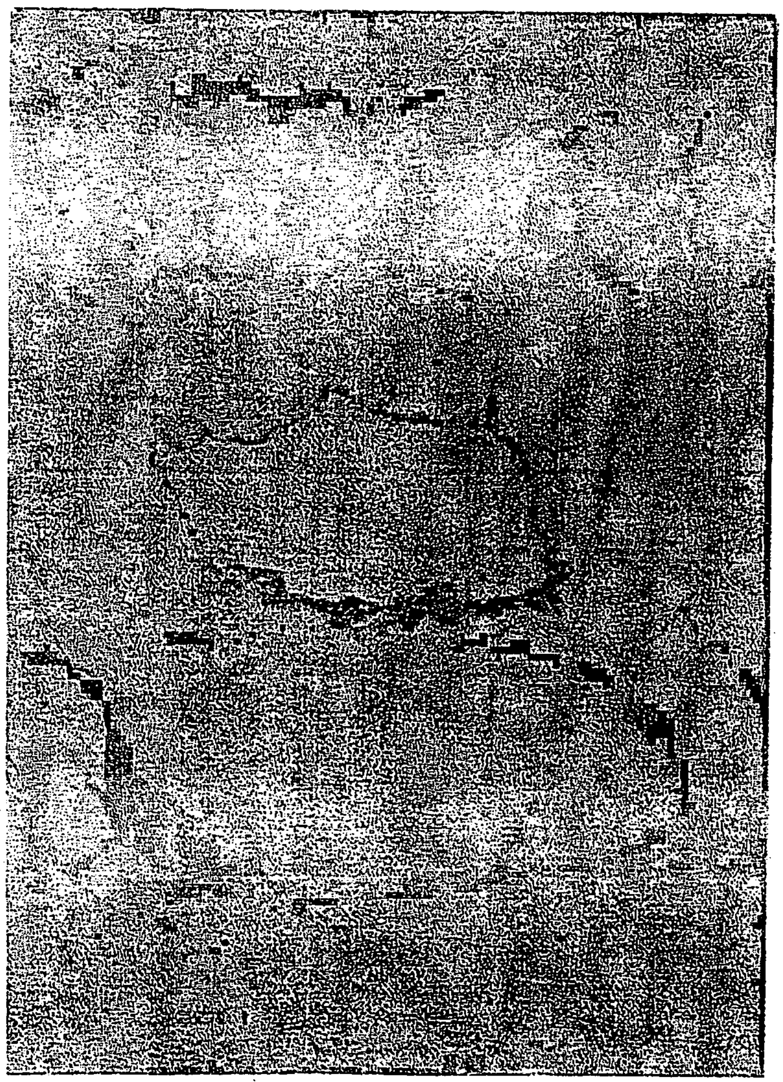


Figure 14

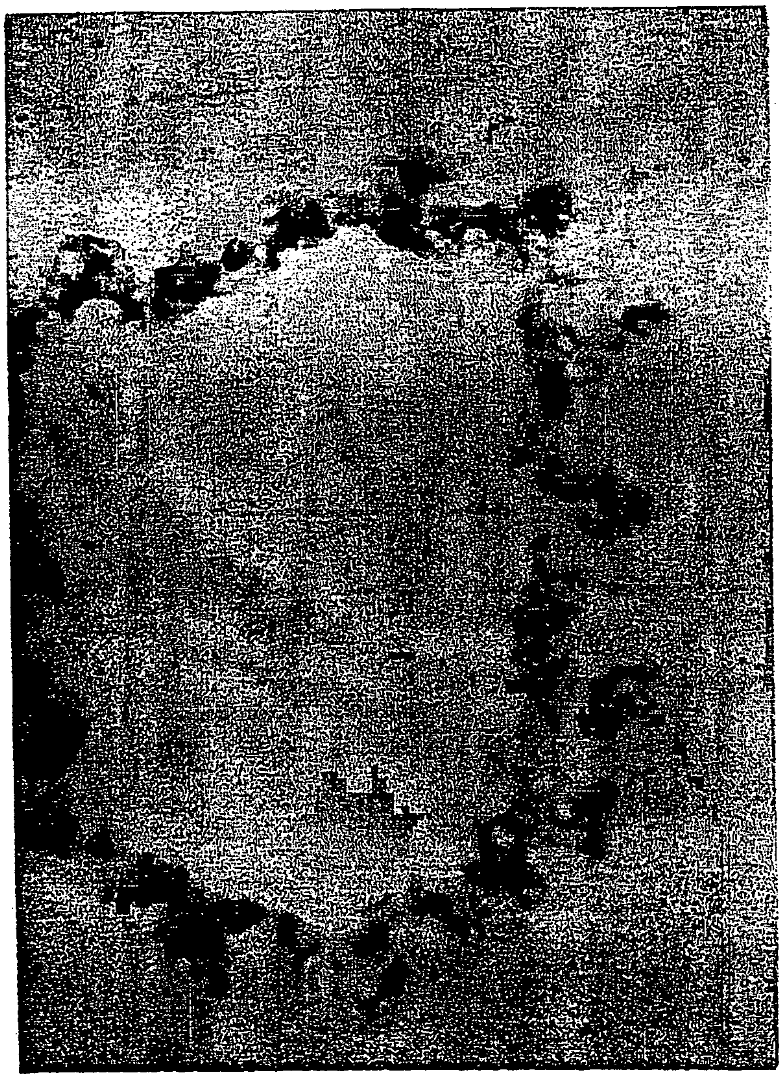


Figure 15

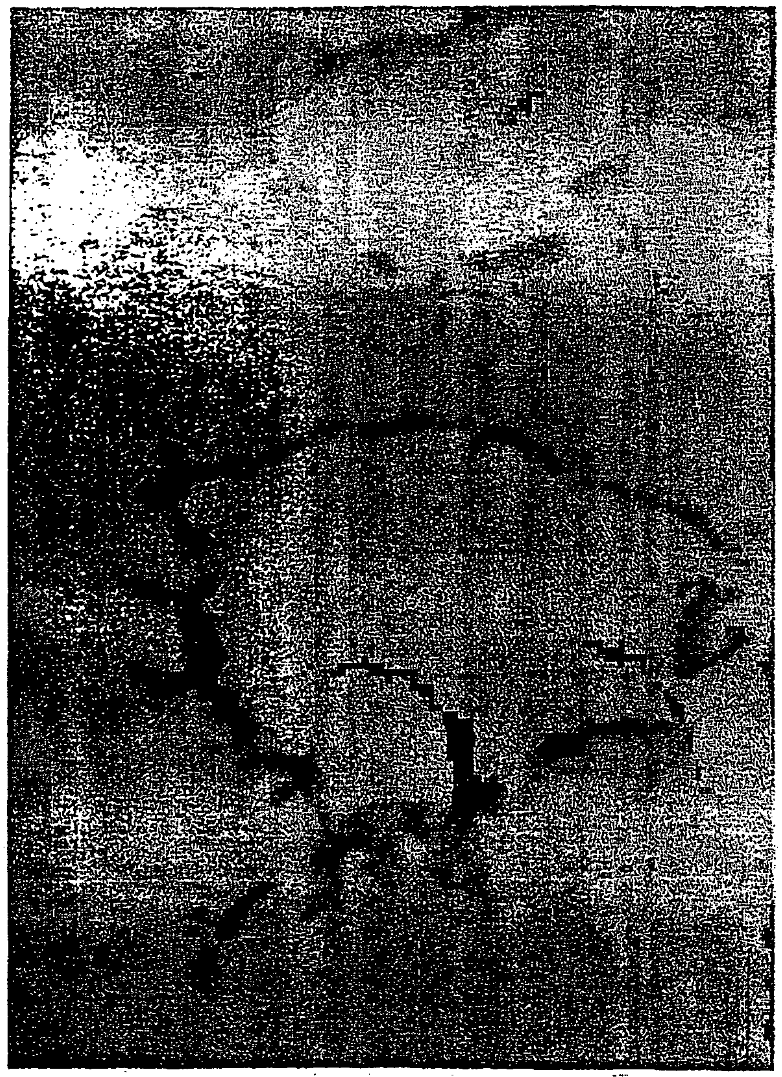


Figure 16

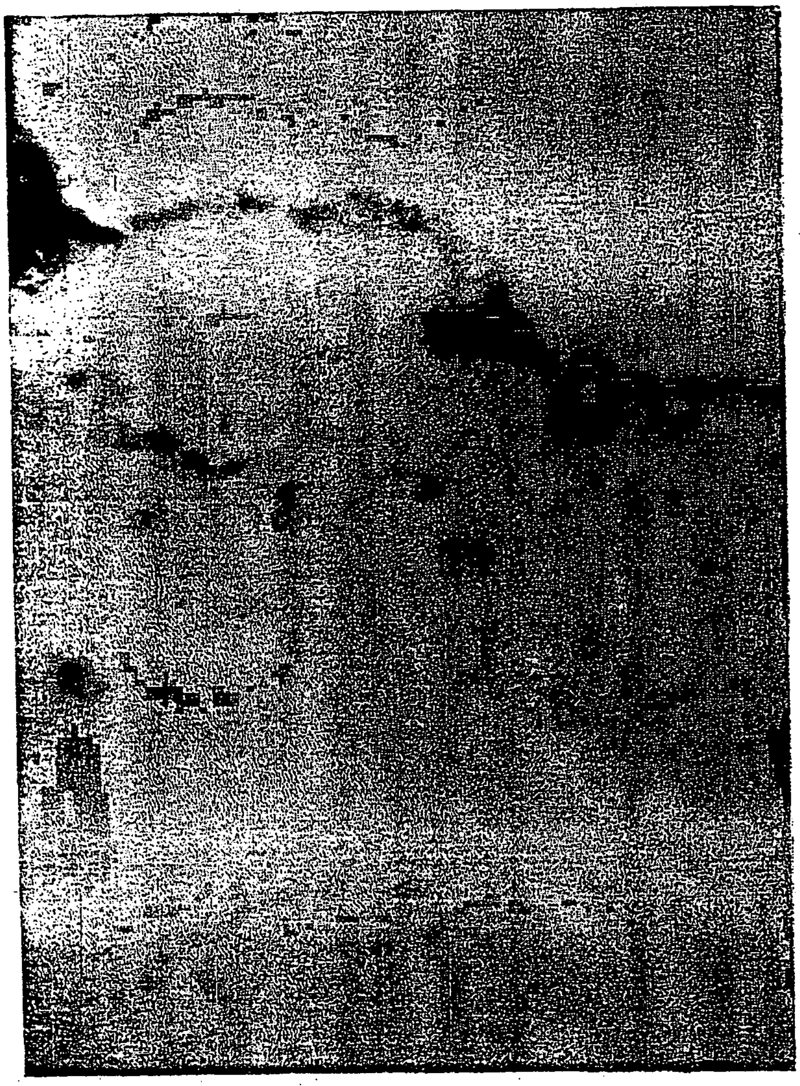


Figure 17

Figure 18

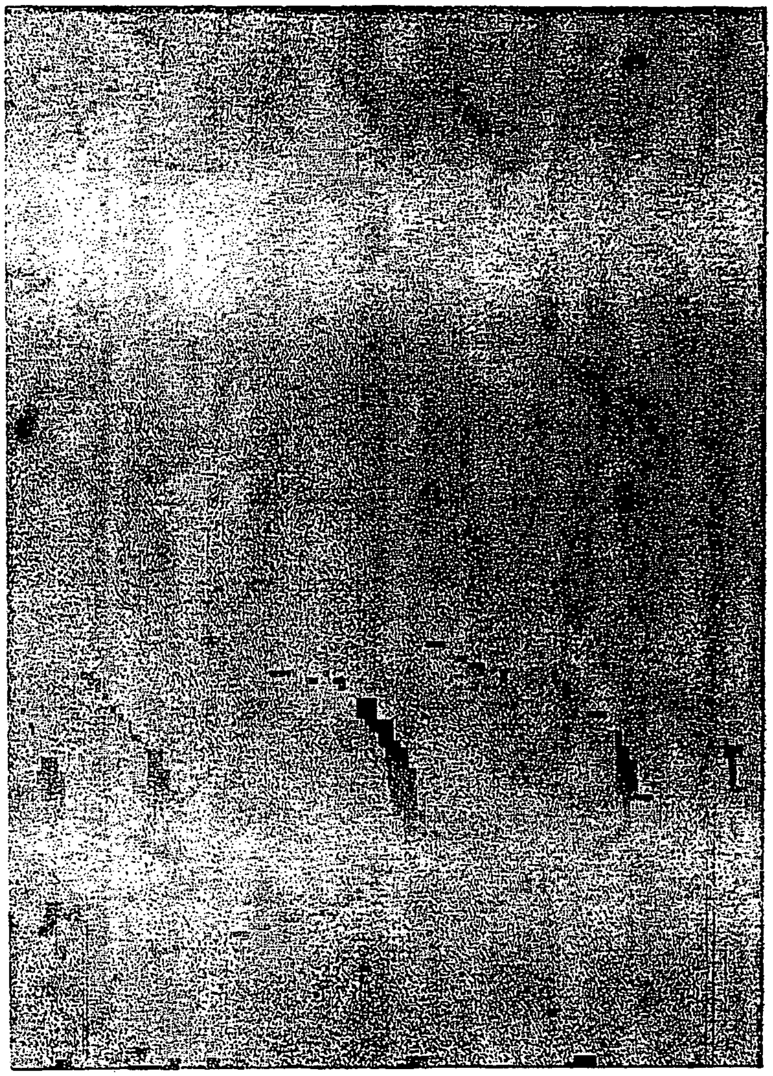


Figure 19

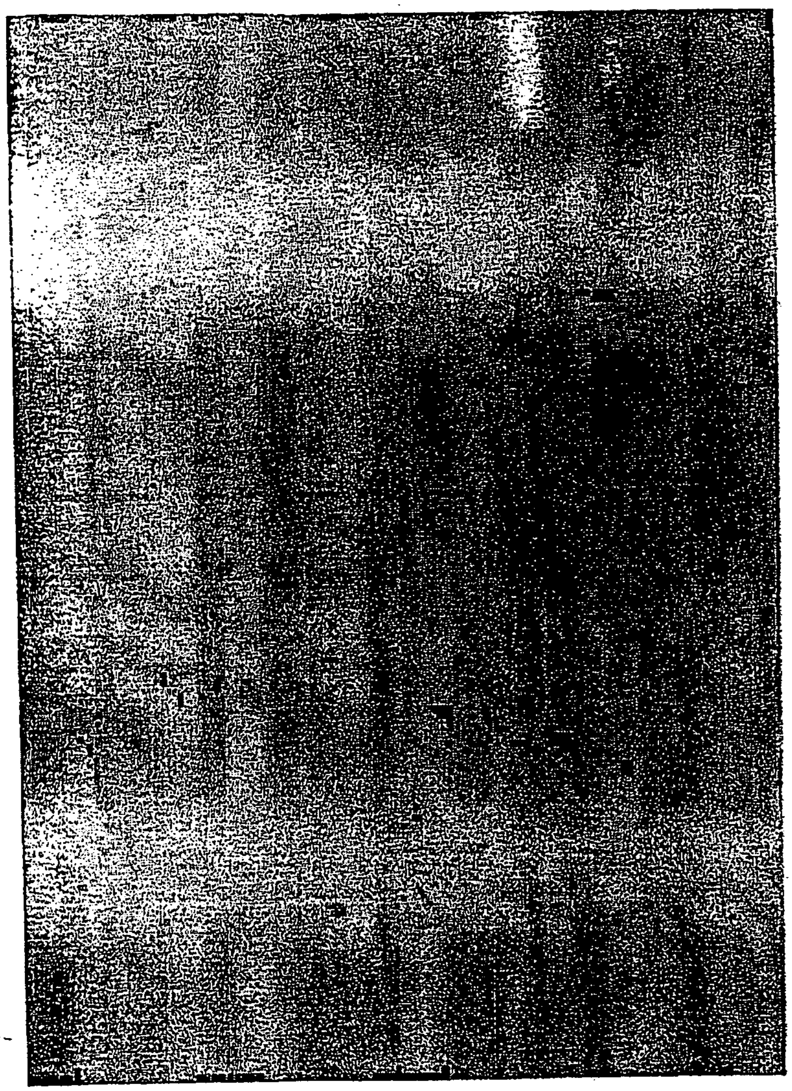


Figure 20

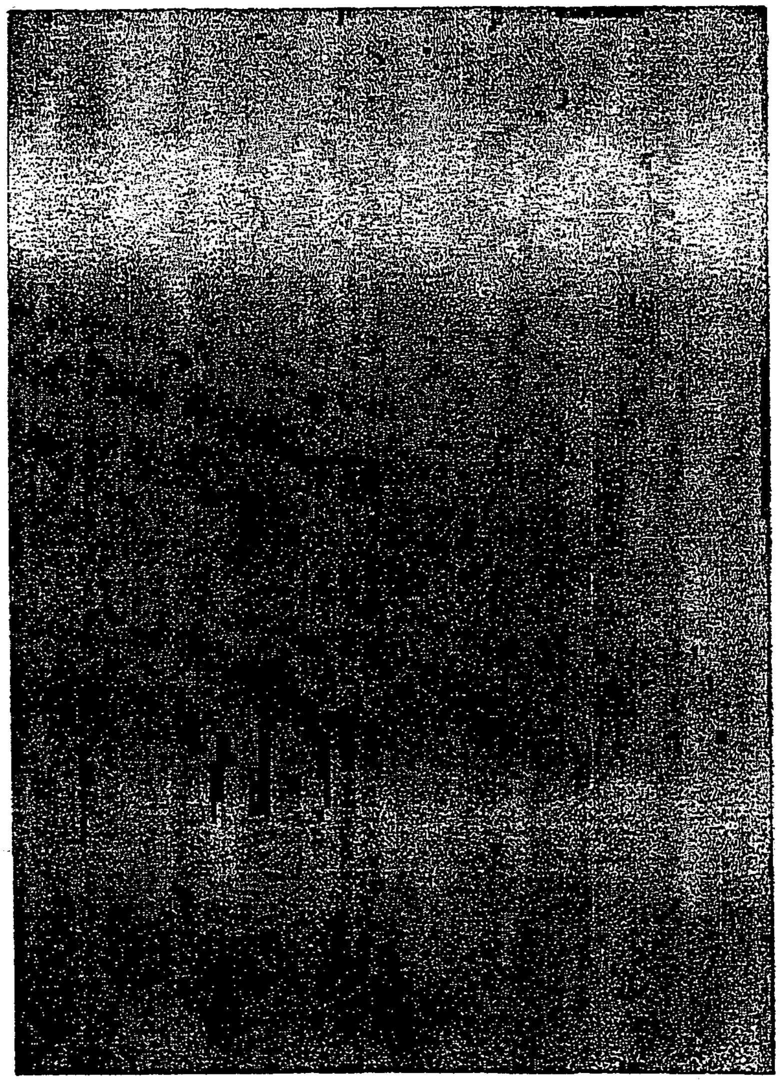


Figure 21

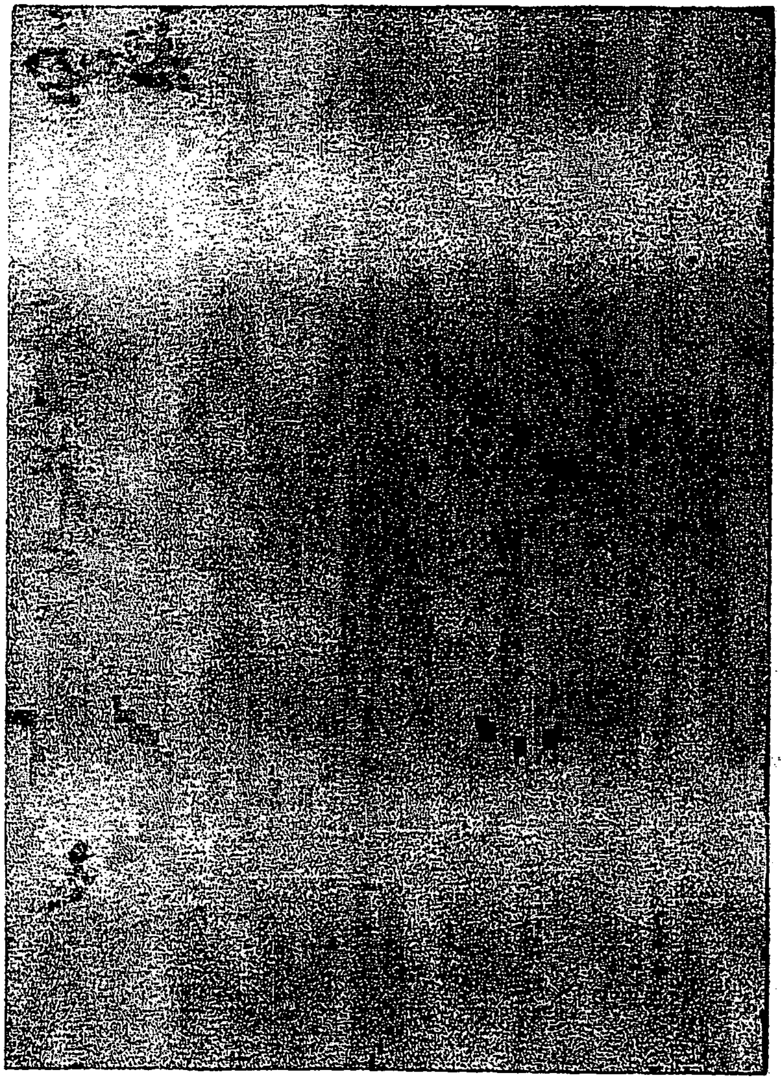
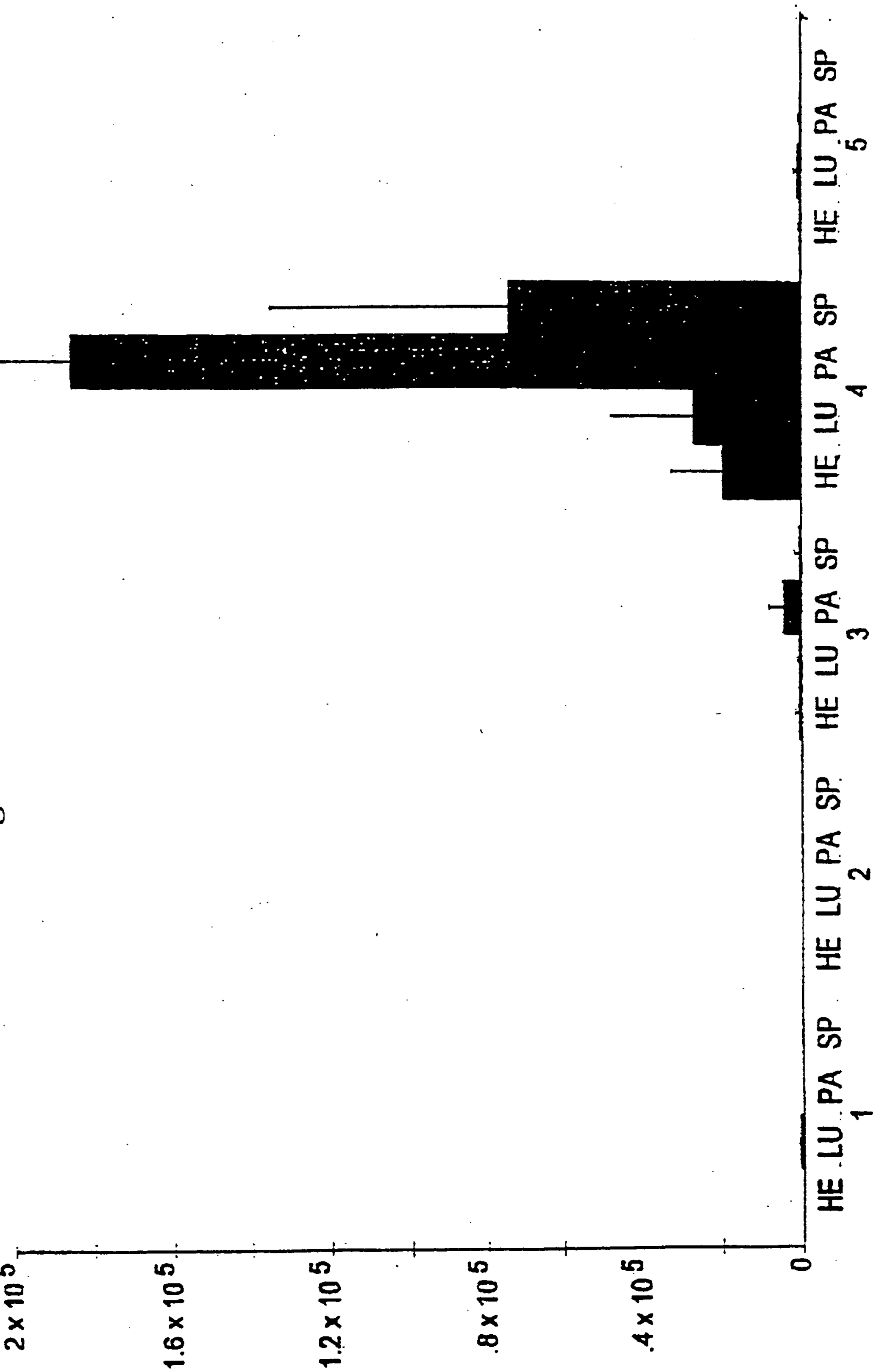


Figure 22

Figure 23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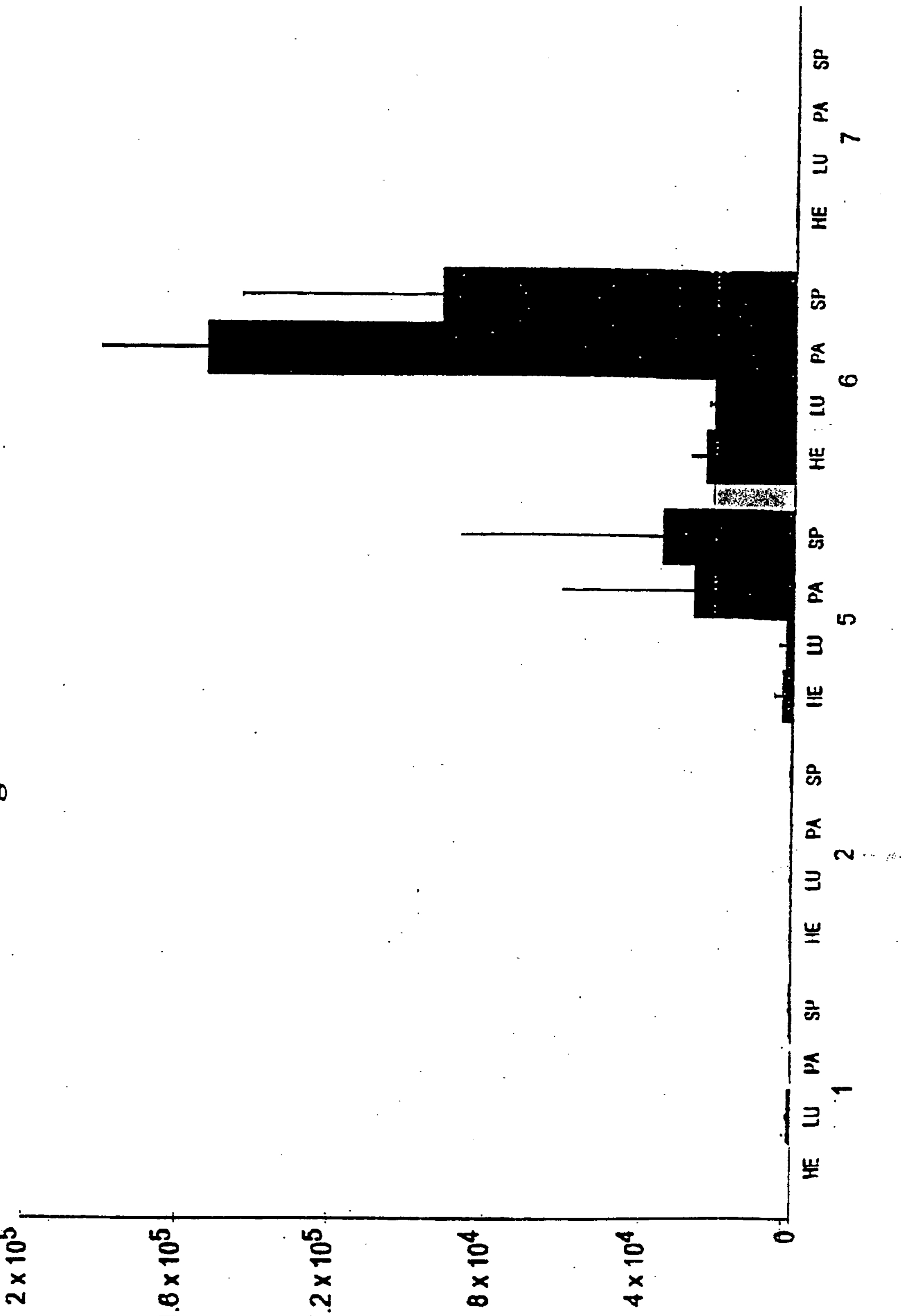
HE LU PA SP
3
HE LU PA SP
4
HE LU PA SP
5

Figure 24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7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09771

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/88 A61K48/00 A61K47/48 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 03709 (VICAL INC.) 4 March 1993 see the whole document ---	1-10
A	WO,A,93 12240 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 24 June 1993 see the whole document ---	1-10
P,X	WO,A,95 26356 (NORTHWESTERN UNIVERSITY) 5 October 1995 see the whole document -----	1-4,9

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 1996	25.10.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gac, G

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/09771

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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